ACHROMOBACTER FISCHERI NITRITE REDUCTASE

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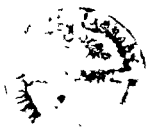

It is certified that the work presented in this thesis has been carried out entirely by the candidate. No part of this work has been submitted for a degree or diploma or other academic award on any previous occasion. The literature concerning the problems investigated has been surveyed and all the necessary references are given. Due acknowledgement has been made whenever the work presented is based on the results of other workers.

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LIST OF ABBREVIATIONS

NAD^+, NADH	Nicotinamide adenine dinucleotide and its reduced form.
$NADP^+$, NADPH	Nicotinamide adenine dinucleotide phosphate and its reduced form.
FMN, $FMNH_2$	Flavin mononucleotide and its reduced form.
FAD, $FADH_2$	Flavin adenine dinucleotide and its reduced form.
BV, BVH	Benzyl viologen and its reduced form.
MV, MVH	Methyl viologen and its reduced form.
DEAE-cellulose	Diethylaminoethyl-ellulose.
EDTA	Ethylenediamine tetraacetic acid.
p-HMB	p-Hydroxymercuribenzoate.
p-CMS	p-Chloromercuribenzene sulfonic acid.
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid).
$NaBH_4$	Sodium borohydride.
SDS	Sodium dodecyl sulfate.
2-ME	2-Mercaptoethanol.
Dansyl chloride	1-Dimethylaminonaphthalene-5-sulfonyl chloride.
Gu.HCl	Guanidine hydrochloride.
S	Svedberg unit (10^{-13}sec) of sedimentation coefficient.
F	Fick unit ($10^{-7}cm^2sec^{-1}$) of diffusion coefficient.
rpm	Revolutions per minute.
O.D.	Optical density.
nm	Nanometer.
µg	Microgram or micrograms.
µmole(s)	Micromole or micromoles.
x g	Times normal gravitational force.
-SH	Sulfhydryl or thiol groups.

-S-S-	Disulfide group.
gm	Gram or grams.
hr	Hour or hours.

C O N T E N T S

Chapter 1

INTRODUCTION

SECTION I

GENERAL ASPECTS OF INORGANIC NITROGEN METABOLISM AND THE SCOPE OF LITERATURE SURVEY

The ultimate source of nitrogen for all forms of life is inorganic nitrogen. From an ecological point of view, all plants and many microorganisms capable of converting the nitrogen atom from its various oxidised states to the level of ammonia and amino groups are ultimately responsible for providing organic nitrogen to the many heterotrophic forms of life. Virtually all animals and numerous microorganisms can fulfill their nitrogen requirement only from exogenous supply of organic nitrogen, and to a lesser extent ammonia, for they lack the biochemical machinery for transforming the more oxidised states of inorganic nitrogen to this level.

The essential features of inorganic nitrogen metabolism revolves about oxidation-reduction reactions. The nitrogen atom has a variety of oxidation states ranging from the disputed oxidation level of plus six as represented by the presumed short half-lived $NO_3(1)$ upto the oxidation level of minus three as represented by ammonia (Table 1). With the exception of the controversial plus six oxidation state, each has been implicated in the inorganic nitrogen metabolism of either intact organism or cell-free preparations. However, of all the oxidation states of nitrogen, nitrate, molecular nitrogen, and ammonia are the most widely distributed in nature.

TABLE 1

OXIDATION STATES OF THE NITROGEN ATOM

Oxidation State	Compound	Hydrate
+ 6	Nitrogen peroxide (NO_3)	Pernitrous acid (H_2NO_4)
+ 5	Nitrogen pentoxide (N_2O_5)	Nitric acid (HNO_3)
+ 4	Nitrogen tetroxide (N_2O_3)	-
+ 3	Nitrogen trioxide (N_2O_3)	Nitrous acid (HNO_2)
+ 2	Nitrogen dioxide: Nitric oxide (NO)	
+ 1	Nitrogen monoxide: Nitrous oxide (N_2O) Nitramide (NO_2NH_2)	Hyponitrous acid ($H_2N_2O_2$)
0	Nitrogen (N_2)	-
-1	Hydroxylamine (NH_2OH)	-
-2	Hydrazine (NH_2NH_2)	-
-3	Ammonia (NH_3)	-

Recently, the scope of interest in biological reduction of nitrate and other nitrogenous oxides had broadened. The current interests can be categorized as follows:

i) Isolation, purification and characterization of enzymes and cytochromes involved in the various reductive steps (2-5).

ii) Identification of electron donors and organic and inorganic components of electron transport chains linked to reduction (6-9).

iii) Determination of mechanisms that control synthesis and functioning of the reductive enzymes (10-12).

iv) Estimation of the agricultural and ecological impact of nitrate reduction on biological interactions in soil, sewage and water (13-15).

Health hazards resulting from the consumption of nitrates or nitrites and economic losses are being considered (16,17). Presently available data applicable to humans whether pertaining to methemoglobinemia, nitrosamine formation or other possible effects of nitrate and nitrite, provide no basis for alarm (16-17).

The literature reviewed in this chapter deals mainly with studies on nitrite reductases from bacteria, fungi and higher plants with a brief reference to nitrate reductase. No attempt has been made to cover exhaustively all the aspects of nitrite reduction. Instead, the emphasis has been placed on those aspects (purification, physico-chemical properties and kinetics) and which form the subject matter of this thesis and also those where substantial progress has been made in recent years.

The recent review by Payne (18) on reduction of nitrogenous oxides as well as those published earlier by Nason and Takahashi (19), Nason (20), Hewitt and Nicholas (21), Takahashi, Taniguchi and Egami (22) and Beevers and Hageman (23) on this subject were of great help in writing this chapter.

S E C T I O N II

METABOLISM OF NITRATE

The biological reduction of nitrate to nitrite occurs in a variety of bacteria and fungi as well as in algae and higher plants. A number of classifications have been proposed for various types of nitrate reduction (19,24-27) but none has been found satisfactory. Jensen (28) suggested five categories according to the products of the reaction whereas Verhoeven (24) differentiated between three types of nitrate reduction, thus: (a) 'assimilation' in which nitrate is reduced only for the elaboration of nitrogenous cell materials, (b) 'incidental dissimilation' in which nitrate acts as a non-essential hydrogen accepter, (c) 'true dissimilation' in which nitrate acts as the essential hydrogen acceptor which enables the organism to grow. An entirely different classification based on the function of the cytochrome system was given by Sato (25). He classified nitrate reducers into three categories (a) Reducers whose cytochromes participate in nitrate reduction; the cytochromes involved, however, differ from species to species. Another

important characteristic common to this group is the strong inhibitory action of oxygen on nitrate reduction. (b) Those whose cytochromes do not participate in nitrate reduction. (c) Those that lack cytochromes. The above three categories suggested by Sato (25) for nitrate reduction were considered by Takahashi et al. (22) as nitrate respiration, nitrate assimilation and nitrate fermentation as illustrated in Table 2. However, it is known that obligate anaerobes can also synthesize cytochromes (29,30). Garret and Nason (31) and Solomonson and Vennesland (32) have concluded that the distinction between assimilatory and respiratory nitrate reduction cannot be made on the basis of the presence or absence of a cytochrome.

Fewson and Nicholas (33) and Nason (20) proposed that nitrate reduction can best be distinguished into two major types: (a) nitrate assimilation or assimilatory nitrate reduction in which the nitrate and its reduction products are reduced to ammonia for the biosynthesis of nitrogen-containing components, and (b) nitrate respiration or dissimilatory nitrate reduction in which nitrate and/or its reduction products serve as the terminal electron acceptors in place of oxygen, usually under anaerobic or partially anaerobic conditions. Depending upon the organisms and its environment, nitrate may be reduced only to nitrite or in a series of steps to more reduced forms of nitrogen. If molecular nitrogen, nitric oxide or nitrous oxide is the product of nitrate reduction, the process is called denitrification. The respiratory or dissimilatory nitrate reduction is inhibited by oxygen. Because of this obvious physiological and enzymological similarity to oxygen respiration,

TABLE 2

CLASSIFICATION OF NITRATE-REDUCING SYSTEMS

Category	Electron transport system	Pathway of reduction	Source
I Nitrate respiration (facultative anaerobes)	Participation of cytochromes (particulate fraction)	1. Nitrate→nitrite (nitrite accumulating reaction)	*E. coli*, *C. diphtheriae*, *S. aureus*, *S. marcescens*
		2. Nitrate→nitrogen (denitrification)	Denitrifiers
		3. Nitrate→ammonia (ammonia-producing reaction)	*B. pumilis*, *Micrococcus*
			B. licheniformis
II Nitrate assimilation (aerobes)	No participation of cytochromes (soluble molybdoflavoprotein)	Nitrate→ammonia ↓ protein	*B. subtilis*, yeast, *Azotobacter*, *E. coli*, *Neurospora* Soybean leaves
III Nitrate fermentation (obligate anaerobes)	No cytochromes	Nitrate→nitrite	

it would be expected that nitrate respiration involves energy-yielding reactions which under given conditions are necessary for the growth and well-being of the organism. One step reduction of nitrate and nitrous oxide is known to support the growth of various bacteria (20,34,35). However, only nitrate reduction is reportedly linked to oxidative phosphorylation (36-38). There are as yet no reports of oxidative phosphorylation coupled specifically to nitrous oxide reduction (18).

Pichinoty (39-41) reported the existence of two types of nitrate reductases, A and B, in nitrate reducing bacteria which differ in some of their properties particularly in their behaviour towards chlorate. Chlorate is a substrate of A and an inhibitor of B. He classified nitrate reducing bacteria into three groups in accordance with their possessing A,B and A and B. Nitrate reductase A, in general, belongs to the respiratory type and is membrane-located. Its formation is induced by nitrate and repressed by oxygen. The enzyme B (soluble), depending upon the species, has assimilatory (Pseudomonas putida, Micrococcus denitrificans) or respiratory (Providentia alcalifaciens Aeromonas hydrophila) function (42). In the former case, it is constitutive and not repressed by oxygen whereas in the latter, it is induced in the presence of nitrate and repressed by oxygen. Several species, however, are known that do not produce enzyme B but nonetheless assimilate nitrate nitrogen.

Two types of enzyme B are now discernible in several species of bacteria. Enzyme B_{α} is activated by IM NaCl, KCl or

CsCl whereas B_p is not (43). A separate chlorate-reducing enzyme, designated enzyme C, and unrelated to nitrate reductase has been reported.

In Escherichia coli, nitrate reductase has a multifunctional character (25,44). It can perform simultaneously two different types of nitrate metabolism, nitrate respiration (anaerobiosis) and apparent nitrate assimilation (aerobiosis). The anaerobic nitrate reduction does not proceed further than the nitrite stage and is profoundly retarded by oxygen, reflecting the character of nitrate respiration. However, it is not known whether the same nitrate reductase performs the two functions. In Aerobacter aerogenes, there is only one nitrate reductase which has a respiratory function under anaerobic conditions, and an assimilatory function under aerobic conditions (41,45). On the basis of differences in factors regulating the synthesis of enzyme that carries out the two different activities, however, Van Triet et al. (46) raised the possibility of two different nitrate reductases in A. aerogenes. In M. denitrificans however, the existence of two nitrate reductases, one having respiratory and the other an assimilatory function, has been reported (47).

Nitrate metabolism in Achromobacter fischeri has been studied in this laboratory and is found unique in that the product of nitrate metabolism is ammonia but both the metabolism of nitrate and the formation of nitrate and nitrite reductases is greatly inhibited by oxygen (48). Achromobacter nitrate reductase involves the participation of bacterial cytochrome

c551 in the electron transport chain. Furthermore, the nitrite reductase of A. fischeri is itself a heme-containing protein (49). In view of the cytochrome participation and the apparent competition by oxygen for the electrons in the conversion of nitrate to nitrite and to ammonia, nitrate metabolism of A. fischeri apparently exhibited the characteristics of the respiratory type (25). However, since the product of nitrate reduction is ammonia, it should belong to the assimilatory type.

S E C T I O N III

NITRATE REDUCTASE

Nitrate reductase catalyzes the reduction of nitrate to nitrite, the first step involved both in the assimilatory as well as respiratory nitrate reduction. Depending upon the type of nitrate reduction carried out by the organism, nitrate reductases can be divided into two major groups (i) assimilatory nitrate reductases and (ii) dissimilatory or respiratory nitrate reductases.

In addition, three different molybdenum-containing enzymes from animal tissues, xanthine oxidase, aldehyde oxidase and xanthine dehydrogenase are also known to catalyze the reduction of nitrate to nitrite, although this is not considered to be their primary function (50,51).

Ivanova and Peiva (52) have recently reported that horseradish peroxidase can also catalyze nitrate reduction at a very high rate using diethyldithiocarbamate and sulfite mixture

as electron donor.

1) Assimilatory Nitrate Reductase

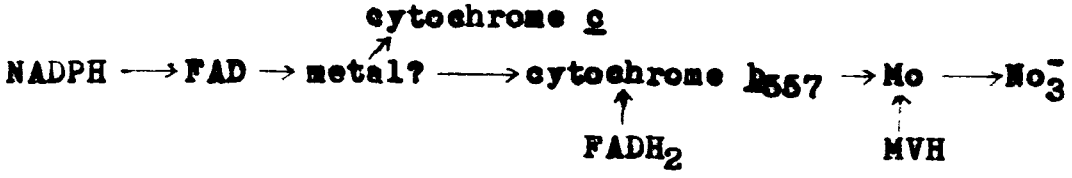
The assimilatory nitrate reductase was first characterized from Neurospora (53) and soybean leaves (54) as a sulfhydryl molybdo-FAD-protein. Both FAD and Mo were shown to function as electron carriers in the following sequence (55).

$$NADPH \longrightarrow FAD \longrightarrow Mo \longrightarrow NO_3^-$$

Oxidation states of molybdenum involved in the oxidation-reduction appear to be +5 and +6 (55,56).

The work of Nason and his colleagues has established several features of the enzymatic apparatus associated with nitrate reduction in N. crassa. N. crassa assimilatory NADPH-nitrate reductase (NADPH: nitrate oxidoreductase, E.C. 1.6.6.2) is a soluble sulfhydryl protein, with FAD, cytochrome b_{557} (N. crassa), molybdenum and an unidentified second metal component as prosthetic groups (31,53,55,57-59). It has a molecular weight of 230,000 (59,60) and displays several other inducible enzymatic activities including FAD-dependent NADPH-cytochrome c-reductase (61), $FADH_2$-nitrate reductase and reduced methyl viologen (MVH)-nitrate reductase (59).

The following pathway of electron transfer in nitrate reduction in N. crassa has been suggested:



Both genetic (60) and biochemical (7) evidence has shown that N. crassa nitrate reductase is composed of at least two subunits. The synthesis of one of these subunits is induced by nitrate. This component is characterized by a capacity to catalyze the reduction of cytochrome c by NADPH. The other subunit, which is a constitutive component of wild-type N. crassa and certain mutants, is characterized by a capacity of catalysis of nitrate reduction by $FADH_2$ or reduced methyl viologen when this subunit is combined with the inducible subunit. Ketchum et al. (7) and Nason et al. (62) have shown that molybdenum-containing constitutive subunit from N. crassa can be replaced by acid-treated molybdenum enzymes from diverse phylogenetic sources extending from prokaryotic through higher eucaryotic organisms. The molybdenum enzymes included bovine milk and intestinal xanthine oxidase, rabbit liver xanthine oxidase and chicken liver xanthine dehydrogenase. Acid-treated preparations of nitrogenase from Clostridium, Azotobacter and soybean bacteroids, liver aldehyde and sulfite oxidases from mammals, plant nitrate reductase and E. coli respiratory nitrate reductase can also replace the constitutive molybdenum-containing subunit of N. crassa (62). By contrast, inorganic molybdate, and certain molybdenum-amino acid complexes as possible catalytic models of nitrogenase failed to yield NADPH-nitrate reductase activity after incubation with the nitrate-induced nit-1 extract (7). Combination of molybdenum-containing subunit from any one of the various sources with the inducible subunit from N. crassa (nit-1) results in reconstitution of functional nitrate

reductase with properties indistinguishable from those of wild-type N. crassa nitrate reductase (7,62). Apparently N. crassa nitrate reductase and the various molybdenum-containing enzymes share highly similar protein subunit. In explaining these results, Nason et al. (62) postulated that the constitutive component is a molybdenum cofactor. Ketchum and Swarin (63) have recently reported that the loss of the gene product in N. crassa (nit-1) can be replaced by a trypsin and protease-insensitive dialyzable component which is present in the extracts of bacteria that are capable of metabolizing dinitrogen and/or nitrate. The component is presumed to contain molybdenum and can probably be viewed as a cofactor as postulated by Nason et al. (62). Evidence for possible existence of common genes affecting both nitrate reductase and nitrogenase has also been obtained in studies with mutants of Rhizobium (64). In more recent papers Nason and his colleagues (65,66) have demonstrated using radioactive molybdenum, the partial reactivation effect specifically by salts and other derivatives of the metal showing that molybdenum (presumably as a component of a larger molecule or cofactor in the in vitro formation of the enzyme) is contributed solely by Neurospora extracts other than that of nit-1, and by acid-treated molybdenum enzymes.

Information is yet scarce on nitrate reductase from nitrate-reducing yeasts. Preliminary experiments with Hansenula anomala (67,68), Candida utilis (69) and Torulopsis nitratophila (70) have indicated that the enzyme resembles

Neurospora nitrate reductase in that it is a metalloflavoprotein specific for NADPH as electron donor.

The presence of assimilatory nitrate reductase has been reported in algae and a variety of higher plants. The nitrate reductase from algae and higher plants has been characterized in recent years (23,71,72).

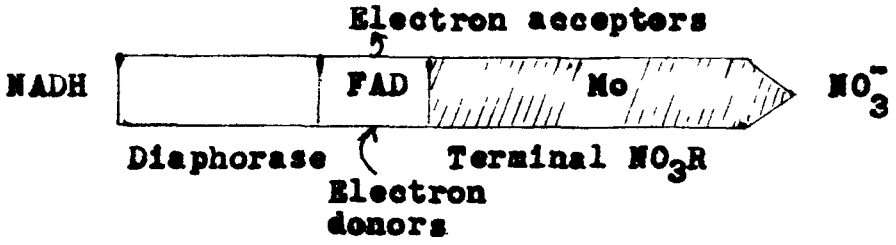
The enzyme is a molybdoflavoprotein and specifically requires NADH as electron donor for the reduction of nitrate to nitrite. The indispensable nature of molybdenum as a component of nitrate reductase from algae and higher plants has been convincingly demonstrated by the use of tungston as a specific inhibitor (76-77) and by other ways (6,73,74). By using ^{185}W, it was possible to get, *in vivo*, a radioactive nitrate reductase-W complex from spinach (77) and *Chlorella* (75) which maintained unaffected its NADH-diaphorase activity but was completely inactive as nitrate reductase. The association of ^{185}W with nitrate reductase was shown to be a weaker than that of Mo. The pathway of electron transfer within the nitrate reductase complex, as suggested by Schrader *et al.* (78), envisages a transfer of electrons from NADH to a flavin moiety and then to molybdenum which ultimately reduces NO_3^- attached at the active site of the enzyme.

Exceptions to pyridine nucleotide specificity are known in algae. Thus, nitrate reductase from *Cyanidium caldarium*, *Dunaliella tertiolecta* and *Ankistrodesmus braunii* can accept electrons from both NADH and NADPH even at the highest degree of purification (79,80). The apparent ability of partially purified

enzyme from leaves of soybean, maize and foxtail to utilize NADPH was shown to be due to the presence of a phosphatase which readily converted NADPH to NADH (81).

In analogy with the enzymes from fungi, the partially purified nitrate reductase from several algae has associated cytochrome c-reductase activity and contains cytochrome b_{557}, in addition to FAD and Mo (18). No involvement of cytochrome could be demonstrated for nitrate reductase from higher plants (82).

Working with highly purified nitrate reductases from spinach, small calabash and Chlorella, Losada et al. (83) concluded that nitrate reductase molecule consists of two chemically separable moieties:a FAD-dependent NADH-diaphorase which can use several oxidised compounds such as cytochromes as electron acceptors, and terminal nitrate reductase which used reduced flavin nucleotides and viologen dyes as electron donors. The two activities participated sequentially in the transfer of electrons from NADH to nitrate as described below:



Intracellular location of nitrate reductase in green plants has been studied. The enzyme was shown to be present in cytosol (84,85). Studies with non-green tissues, however, suggested that nitrate reductase is a soluble (cytoplasmic)

enzyme (86).

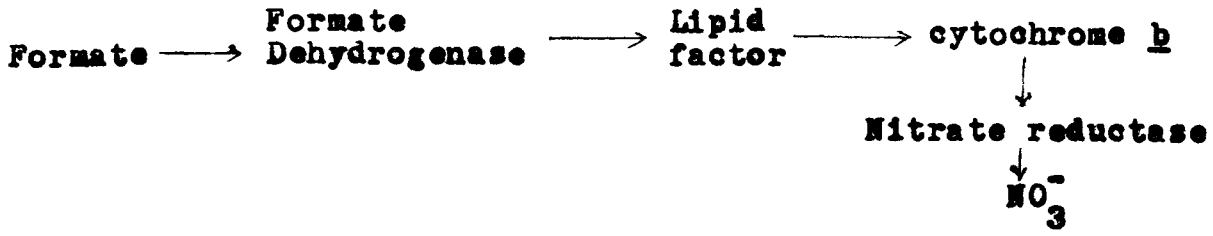
By contrast with nitrate reductase from the plant kingdom, information is yet very scanty on nitrate reductases from the assimilatory nitrate-reducing type bacteria. Nicholas and Mason (87) purified a soluble NADH-linked nitrate reductase from E. coli strain B; the enzyme was a metalloflaveprotein with FAD as the prosthetic group and molybdenum as a probable metal constituent. Taniguchi and Ohmashi (88) isolated an inducible, particulate NADH-specific nitrate reductase from Azetobacter vinelandii which was characterized as a sulfhydryl metalloenzyme; the activity of the enzyme was stimulated about 2-fold by added FAD or FMN. An assimilatory nitrate reductase has recently been reported from Azotobacter chroococcum (89). The enzyme (M.W., 100,000) was characterized as a molybdo-protein which could use only reduced viologen dyes as electron donors. The enzyme did not accept electrons from reduced pyridine nucleotides.

ii) Respiratory Nitrate Reductase

In contrast to assimilatory nitrate reductases which are often found in the soluble cytoplasmic fraction, the respiratory nitrate reductases are, in general, membrane-bound. Exception to this generalization are found, as exemplified by the soluble respiratory nitrate reductase of Spirillum itersonii (90).

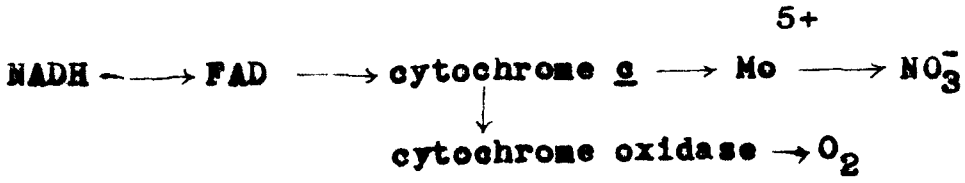
Taniguchi and Itagaki (91) isolated a particulate nitrate reductase system from E. coli which included cytochrome b as an intermediary electron carrier from formate or NADH to nitrate; this system possessed remarkably high activity of

nitrate reductase. The best natural electron donors for nitrate reduction by the intact particle were $FMNH_2$, $FADH_2$, formate and NADH, whereas reduced methyl and benzyl viologens acted as best artificial electron donors. The nitrate reductase, the terminal enzyme of the particulate system was solubilised and purified to a homogeneous state. The enzyme was shown to have a molecular weight of one million and contained one atom of bound Mo and about 40 atoms of iron per molecule but no bound flavin or cytochrome. The solubilised enzyme utilized reduced methyl viologen as electron donor but formate and NADH were no longer effective. The data suggested that reduced cytochrome b would be the favourable electron donor for nitrate reduction. Itagaki, Fujita and Sato (92) presented evidence indicating the involvement of cytochrome b as electron donor. The following pathway for the transfer of electrons was suggested:

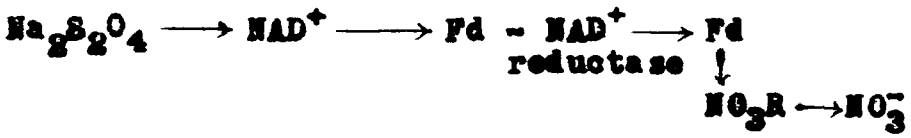


The lipid factor could be replaced by vitamin K.

Fewson and Nicholas (33) reported a NADH-specific nitrate reductase from denitrifying cells of *Pseudomonas aeruginosa* which contained FAD, cytochrome c and Mo as functional components. The following scheme for electron transport was suggested:

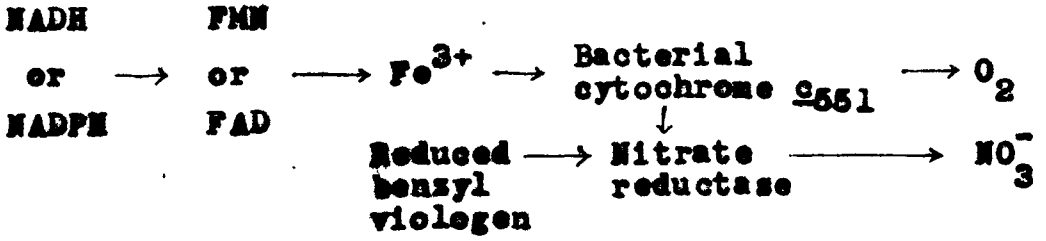


The respiratory nitrate reductase from Micrococcus denitrificans was purified and characterized by Lam and Nicholas (93). The enzyme was molybdoprotein but did not contain cytochrome or flavin. NADH, $FADH_2$, $FMNH_2$ succinate and reduced cytochrome could not donate electrons to the enzyme. Only reduced benzyl viologen and methyl viologen were utilized as electron donors. The purified preparation of the nitrate reductase obtained by Forget (4) from the same organism did not accept electrons from NADH, but reduced flavin nucleotides were effective electron donors. The enzyme was characterized as a non-heme iron protein which contained only traces of Mo. The involvement of iron and Mo as functional components of nitrate reductase was later confirmed on the basis of EPR studies (5). Chiba and Ishimoto (94) reported a nitrate reductase from Clostridium perfringens that reduces nitrate to nitrite or ammonia. Ferredoxin was found to be an intermediary electron carrier in nitrate reducing system with NADH as the electron donor. The following pathway for electron transport was suggested:



Sadana and McElroy (95) purified and characterized a nitrate-reducing system from A. fischeri and proposed the

following pathway of electron transfer:



The electron transport chain was separated into two soluble fractions: (a) the electron donor system, namely a NAD(P)H-cytochrome c-reductase with a requirement for FAD or FMN; and (b) the terminal nitrate reductase which mediated the transfer of electrons from reduced cytochrome to nitrate. When reduced benzyl viologen supplied the electrons, the bacterial cytochrome was not involved. On further purification, nitrate reductase free from cytochrome component was obtained by Sadana et al. (96) and the absorption bands at 550 nm, 520 nm and 419 nm observed in the reduced state (95) were shown to be due to cytochrome impurities. Ultracentrifugal studies indicated that the A. fischeri nitrate reductase was a much smaller molecule than the enzyme from E. coli reported by Taniguchi and Itagaki (91).

Knook and Planta (97) have shown that NADH-dependent nitrate reductase involved in respiratory reduction of nitrate in A. aerogenes requires ubiquinone-8 and cytochrome b, isolated from the same organism, as electron carriers.

Cytochrome-linked nitrate reductases have also been purified, though not thoroughly characterized, from Nitrobacter agilis (98), Rhizobium japonicum (99), Bacillus

stearothermophilus (101) and Bacillus cereus (102).

iii) Nitrate Respiration and Cytochromes

There have been numerous reports of enhanced cytochrome synthesis in various organisms growing with nitrate or nitrite under conditions when nitrate acts as an alternate electron acceptor to oxygen. With denitrifying bacteria such as M. denitrificans, considerable amounts of soluble cytochrome c are formed by cells grown anaerobically with nitrate (103,104). Soluble forms of the pigments are also found in Haemophilus parainfluenzae (105), E. coli (106) and S. itersonii (107) when these organisms are grown anaerobically with nitrate. An aerobic nitrate-dependent growth of M. denitrificans (104,108) and R. japonicum (109) represses synthesis of a + a_3 cytochromes characteristically produced by well-aerated cells. Moreover, increase in the production of c-type cytochrome, Rhizobium haemoglobin and cytochrome P_{450} are concomitant with increases in the synthesis of nitrate and nitrite reductases in the cells. Highly purified preparations of two-heme (c-, and d-type) cytochromes associated with nitrite reduction have been obtained from M- denitrificans (110), P. aeruginosa (111) and Alcaligenes faecalis (112). A copper-containing cytochrome c_{552} isolated from Pseudomonas denitrificans was shown to couple lactate oxidation to the reduction of nitrate to nitric oxide (34). Stimulation of nitric oxide production from nitrite in Pseudomonas perfectomarinus has also been observed by a specific cytochrome c_{548} isolated from the same organism (113).

S E C T I O N IV

NITRITE REDUCTASE

The term nitrite reductase is used for all enzymes that catalyze the reduction of nitrite regardless of the end product of reduction (114). Nitrite is the first stable intermediate in the nitrate reduction by plants, fungi and bacteria.

1) Occurrence

Nitrite reductases are widely distributed in nature. The enzyme has been reported from bacteria, fungi, algae and higher plants.

Higher plants

The enzyme nitrite reductase, which ctalyzes the reduction of nitrite to ammonia (23,82,115), is widespread in higher plants. Enzymatic reduction of nitrite was first described briefly by Nason et al.(116); soybean leaf extract catalyzed ammonia formation from nitrite in presence of either NADH or NADPH and manganese ions. Similarly, in a brief report Vaidyanathan and Street (117) reported NADH-depdendent disappearance of nitrite as ammonia in tomato extracts. Stoichiometry of the disappearance of nitrite and ammonia formation was established for the first time by Hageman et al. (118). Photosynthetic nitrite reduction was reported in wheat leaves by Vanecko and Varner (119) but the reaction product was not identified. The participation of photosynthetically reduced electron donor in nitrite reduction was reported in a similar grana system of tomato (120). Subsequently several

groups demonstrated that ferredoxin was the physiological electron donor (121-123). This non-heme iron protein replaced viologen dyes as the electron carrier in the dark (124,125). Since then a number of groups have attempted to isolate and purify nitrite reductase from higher plants (23,126-128).

Studies of the intracellular location of nitrite reductase in green tissues were carried out and most workers seem to agree that the enzyme occurs in chloroplast fraction after aqueous or non-aqueous isolation (23,84-86). In non-green tissues nitrite reductase was found to be localized in the stroma of the chloroplast (86).

Algae

Photochemical reduction of nitrite was first observed by Kessler (129) in cultures of Ankistrodesmus. He later demonstrated that nitrite served as an efficient Hill reagent for oxygen evolution of Scenedesmus braunii and that light immediately stimulated nitrite reduction (130). Huzisige and Satoh (131) observed a similar light dependence of nitrite reduction by Euglena gracilis. In the blue-green alga Anabaena cylindrica the photochemical reduction was first demonstrated by Hattori (132) who also showed nitrite reduction with molecular hydrogen as electron donor (133). A soluble nitrite reductase was isolated and purified from cell-free extracts of D. tertiolecta (134). The enzyme resembled nitrite reductase of higher plants in that it was a ferredoxin-dependent enzyme. More recently, Zumft (135) obtained electrophoretically homogeneous enzyme from Chlorella fusca which

requires ferredoxin, reduced chemically, photosynthetically or by reconstituted enzymatic systems, as its natural electron donor.

Fungi

In contrast to ferredoxin-nitrite reductase from algae (135) and higher plants (136), which has been purified to homogeneity and its composition and properties studied in detail, nitrite reductase from fungi has been only superficially examined to-date. Nason et al. (116) partially purified from N. crassa (and from soybean leaves) a nitrite reductase which catalysed the reduction of nitrite to ammonia via hydroxylamine by reduced pyridine nucleotides. The enzyme had a specific FAD requirement as well as a metal component. Nicholas et al. (137) further purified the N. crassa nitrite reductase and concluded that it was NADH-dependent and contained FAD, Fe, Cu and -SH groups. Studies on the regulation of nitrite reductase in Aspergillus nidulans (138) and N. crassa (139, 140) have shown that the enzyme is repressed by ammonia and induced by nitrate and nitrite. More recently Rivas et al. (70) isolated and purified from T. nitratophila a NAD(P)H-dependent nitrite reductase that specifically required FAD and some metal component.

Bacteria

The dissimilatory nitrite reduction was first reported by Yamagata (141) in a cell-free preparation of Bacillus pyocyaneus. Denitrifying-type nitrite reductases which catalyzed the reduction of nitrite to nitric oxide was subsequently reported from Thiobacillus denitrificans (142)

P. stutzeri (143), Bacillus subtilis (144), P. aeruginosa (145) and E. coli, strain K12 (114) grown anaerobically in the presence of nitrate. Yamanaka, Ota and Okunuki (146,147) obtained a cytochrome oxidase from P. aeruginosa which functioned as a nitrite reductase under anaerobic conditions. The existence of a nitrite reductase which reduced nitrite to nitrogen was reported by Asano (148) in an aerobic denitrifier, a halotolerant Micrococcus (strain 4203). Newton (110) isolated a cytochrome from M. denitrificans which functioned as a nitrite reductase and was shown to contain two hemes (heme c and heme d). In recent years respiratory nitrite reductases have been isolated and purified from Corynebacterium nephridii (149) A. faecalis (112), Achromobacter cycloclastes (150) and P. perfectomarinus (10) grown under denitrifying conditions. Finally an ammonia-oxidising bacteria, Nitrosomonas europaea which ordinarily generate nitrite was shown to contain a hydroxylamine-dependent nitrite reductase that catalysed nitrite reduction to nitric oxide (151).

The partially purified preparation of hydroxylamine oxidase from the same organism was shown by Ritchie and Nicholas (152) to have retained nitrite reductase activity. This raised doubts as to the identity of nitrite reductase in N. europaea. Recently, however, the same authors reported the separation of nitrite reductase from hydroxylamine oxidase (153). Evidence was presented for the probable identity of nitrite reductase as a copper protein.

By contrast with nitrite reductases from the plant kingdom, information on nitrite reductases from bacteria of the assimilatory nitrate-reducing (non-denitrifying) type is very scanty (20-22). The assimilatory nitrite reduction was first observed by Taniguchi et al. (154) in Bacillus pumilis grown aerobically in the presence of nitrate. Assimilatory nitrite reduction also occurs in Azotobacter species (155,156), Clostridium pasteurianum (157), Rhodospirillum (158), and in soil actinomycetes (159). E. coli strain Bn grown in deep standing cultures with nitrate as the sole source of nitrogen has been shown to contain at least three nitrite reductases that reduced nitrite to ammonia (160) but only the enzyme specific for NADH appeared to be responsible for physiological nitrite reduction (161).

ii) Purification

Bacteria

The purification of bacterial nitrite reductases has been attempted from a number of sources. Nitrite reductases thus far prepared from P. stutzeri (143), P. aeruginosa (111, 145, 162), P. denitrificans (163), E. coli Bn (161), E. coli K-12 (114), M. denitrificans (110) N. europaea (151), A. faecalis (112) and A. cycloclastes (150) were of various degrees of purity.

The purification procedures which result in highly purified bacterial nitrite reductases are briefly described below.

Pseudomonas aeruginosa

The water soluble nitrite reductase which also had cytochrome oxidase activity was extracted and purified by Horio et al. (111). The purification procedure consisted of rivanol treatment, chromatography on an Amberlite IRC-50 or Doulite CS-101 column, ammonium sulfate fractionation and zone electrophoresis on a vertical starch gel column. The purity of the best preparation as judged by diffusion and sedimentation patterns was approximately 70%.

Yamanaka et al. (162) modified the procedure of Horio et al. (111) and obtained a crystalline preparation of the enzyme. The chromatography was performed on Amerlite CG-50 column and the last step, the zone electrophoresis on vertical starch gel, of the earlier procedure (111) was deleted. Instead the enzyme obtained after ammonium sulfate fractionation was crystallized with ammonium sulfate. The homogeneity of the preparation by other criteria was not mentioned.

Micrococcus denitrificans

A purification procedure yielding a highly purified nitrite reductase of M. denitrificans has been reported by Newton (110). The enzyme was purified by a sequence of steps involving preparation of crude cell-free extracts by passing the cell suspension twice through a modified French Press, treatment with DNase and addition of ammonium sulfate to 30% saturation. The further purification was carried out by treatment with DEAE-cellulose, gel filtration on Sephadex, and chromatography on hydroxylapatite gel column. The purified enzyme was 99% pure

as judged by its behaviour on cellulose acetate and polyacrylamide gel electrophoresis.

Pseudomonas denitrificans

The procedure consisted of ammonium sulfate fractionation, column chromatography on Amberlite CG-50 and carboxymethyl cellulose (143). The enzyme was found homogeneous in the ultracentrifuge.

Alcaligenes faecalis

Iwasaki and Matsubara (112) described a purification procedure which results in a crystalline preparation of the enzyme from cell-free extracts of A. faecalis. The cell-free extracts were subjected to chromatography on Amerlite CG-50, ammonium sulfate fractionation, gel filtration on Sephadex G-200, fractionation on hydroxylapatite column followed by crystallization with ammonium sulfate. The purified enzyme showed a small amount of impurity on disc gel electrophoresis.

Achromobacter cycloclastes (150)

The soluble fraction of the crude extract which contained most of the nitrite reductase activity was applied to Amberlite CG-50 column. The active fraction which remains unadsorbed on the column was subjected to chromatography on DEAE-cellulose, ammonium sulfate fractionation, gel filtration on Sephadex G-150 and precipitation with ammonium sulfate at 90% saturation. The partially purified enzyme was again subjected to gel filtration on the same column, DEAE-cellulose chromatography and ammonium sulfate fractionation. The purified enzyme was found to be slightly contaminated with colourless

proteins as revealed by polyacrylamide disc gel electrophoresis.

Fungi, Algae and Higher Plants

Nason et al. (116) purified Neurospora nitrite reductase by fractionation with ammonium sulfate and calcium phosphate gel. They achieved only 10-fold increase in the specific activity. A 50-fold increase in specific activity was achieved by Nicholas et al. (137). The clear supernatant was fractionated with calcium phosphate gel and ammonium sulfate. Nitrite reductases have also been purified from spinach (122,124,126,164), maize (124), vegetable marrow leaves (127,165), A. cylindrica (125) and D. tertiolecta (134) and the enzyme obtained was of various degrees of purity. More recently, Cardenas et al. (136) and Zumft (135) have obtained electrophoretically homogeneous enzymes from spinach and C. fusca respectively. The purification procedure of the enzyme from spinach and Chlorella are discussed briefly:

Chlorella fusca (135)

The crude extract obtained by homogenizing the algal cells and centrifugation was subjected to a fractionation procedure comprising of chromatography on DEAE-cellulose, ammonium sulfate precipitation, gel filtration on Sephadex G-100 and a second chromatography on DEAE-cellulose. At this stage, the enzyme showed two bands showing nitrite reductase activity on polyacrylamide gel electrophoresis. The two components were resolved by preparative polyacrylamide gel electrophoresis. The purified enzyme had a specific activity of 51.7 μmoles nitrite reduced per min per mg protein.

Spinach (136)

The crude extract prepared as described by San Pietro and Lang (166) was subjected to acetone precipitation. The precipitate was dialysed and applied to DEAE-cellulose column. The active eluate was fractionated with ammonium sulfate between 45 and 70% saturation and by gel chromatography on Sephadex G-100. The active fractions were concentrated on DEAE-cellulose column and finally purified by preparative electrophoresis on 7.5% polyacrylamide gel column. The purified enzyme was homogenous as judged by disc gel electrophoresis and had a specific activity of 33.85 μmoles nitrite reduced per min per mg protein.

PROPERTIES

iii) Properties

Molecular weight

The molecular weights reported for nitrite reductase from algae and higher plants are in the narrow range of 60,000 - 72,000. A 40-fold pure enzyme from A. cylindrica was reported to have a molecular weight of 68,000 as determined by its elution behaviour on Sephadex G-100 (125). The same technique yielded a value of 61,000-63,000 for a highly purified nitrite reductase from vegetable marrow (165). The molecular weight of highly purified, though not completely homogeneous, spinach enzyme was determined by a number of different methods (164). Thus, gel filtration yielded a value 60,000 whereas a

higher value of 72,000 was obtained from the sedimentation data. The higher value was supported by a subunit molecular weight of 37,000 determined by SDS-polyacrylamide gel electrophoresis. Cardenas et al. (136) reported a molecular weight of 63,000 for the electrophoretically pure spinach nitrite reductase. The same value (63,000) was obtained, using gel filtration technique, for a homogeneous enzyme from C. fusca (135). The nitrite reductase from a green alga, D. tertiolecta was partially purified and shown to have a molecular weight of 70,000 as determined by its elution behaviour on a calibrated Sephadex G-200 column (134).

In contrast to nitrite reductases from algae and higher plants, the molecular weight of bacterial enzyme exhibited a greater degree of variation (67,000-200,000). A number of molecular weight values have been reported for the nitrite reductase from P. aeruginosa. Thus Horio et al. (111), using a 70% pure preparation, reported an approximate value of 90,000 as calculated from the sedimentation coefficient, diffusion coefficient and partial specific volume. From gel filtration, however, the molecular weight obtained was 85,000 (110), whereas a significantly lower value of 67,500 was determined on the basis of amino acid composition by Nagata et al. (167). The higher values obtained by earlier workers were attributed to the fact that the enzyme used in their studies was impure.

Recently, Kuronen and Ellfolk (168) carried out sedimentation studies with a crystalline preparation of Pseudomonas

nitrite reductase and obtained a molecular weight of 119,000 based on the sedimentation data. Their estimates of about 63,000 and 65,000 for SDS-treated and succinylated enzyme, respectively, led them to suggest that the native enzyme consists of two subunits.

Iwasaki et al. (163) estimated from the sedimentation data a molecular weight of 149,000 for the P. denitrificans nitrite reductase, whereas for the M. denitrificans enzyme a molecular weight of 120,000 (110) was calculated from the gel filtration data.

Molecular weights of 90,000 (112), 69,000 (150) and 67,000 (156) were obtained respectively, for the nitrite reductase from A. faecalis, A. cycloclastes and A. chroococcum. The first two estimates were made by the gel filtration technique whereas the latter by sucrose density gradient centrifugation. An unusually high value of about 200,000 was reported by Cox et al. (3) for a partially purified preparation of nitrite reductase from P. perfectomarinus

Sedimentation coefficient, Diffusion coefficient and partial specific volume

Horio et al. (111) carried out sedimentation and diffusion measurements with P. aeruginosa nitrite reductase which was only 70% pure. Sedimentation and diffusion coefficients were determined to be 5.8 S and 5.8 F and were essentially independent of the protein concentration tested (0.5 ~ 1.0%). A value of 0.73 ml per g for the partial specific volume was determined from the density measurements.

Using a modified purification procedure, a crystalline nitrite reductase was reported from *P. aeruginosa* by Kuronen and Ellfolk (168). The native enzyme had a molecular weight of 119,000 and was shown to contain four iron atoms per molecule. The native enzyme dissociated after succinylation or in SDS into two subunits which contained two iron atoms, heme c and heme d. The molecular weight of the subunits, as determined by sedimentation measurements and SDS-polyacrylamide gel electrophoresis, was around 65,000.

More recently, Ida and Morita (164) obtained an ultracentrifugally homogeneous nitrite reductase from spinach. The molecular weight of the native enzyme was determined to be 72,000 from sedimentation and diffusion coefficients. The results of SDS-gel electrophoresis suggested that the native enzyme consists of two subunits of molecular weight of 37,000.

Amino Acid Composition

Amino acid composition of nitrite reductases has been reported in recent years. Nagata *et al.* (167) were the first to report the amino acid composition of a twice crystallized preparation of the enzyme from *P. aeruginosa*. The enzyme was rich in acidic amino acids and contained only two half-cystine residues as revealed by performic acid oxidation. Neither the free sulfhydryls nor the -S-S- were detected in urea and SDS-denatured enzyme. The two half-cystine residues detected were assumed to bind heme-c moieties present in the enzyme.

Zumft (135) has recently reported the amino acid composition of *C. fusca* nitrite reductase. *Chlorella* enzyme

also showed a slight predominence of acidic amino acids which was consistent with its isoelectric point of around 5. The four tryptophan residue determined in the enzyme molecule were in accordance with its ultraviolet absorption. The enzyme was also shown to contain 10 sulfhydryl groups which were shown to be essential for enzyme activity.

N-terminal amino acid residue

The only report of N- terminal determination was that of Nagata et al. (167) who identified lysine as the N-terminal amino acid of P. aeruginosa nitrite reductase. Attempts to quantitate the N-terminal amino acid were unsuccessful as the yield of di (2,4-dinitrophenyl)-lysine was low.

Inhibition Studies with Sulfhydryl Reagents

Nitrite reductases are sensitive (82,114,135,137,145, 156,160,170) to p-HMB (1 μM to 1 mM). The inhibition by p-HMB is reversible in most cases when incubated with 5-100 fold excess of cysteine or reduced glutathione (114,137,145,170,171). The enzyme is also inhibited by phenyl-mercuribenzoate, iodosobenzoate and heavy metals (137,171,172). Iodosobenzoate inhibition was not reversed by reduced glutathione possibly because disulfide bonds were formed by the reaction with this reagent (137). The possible site of action of these reagents was assumed to be sulfhydryls, but no titration studies with purified enzyme were reported and the number of -SH groups involved in enzyme function is not known.

With spinach nitrite reductase, p-HMB (0.1 mM) inhibition was observed only ± when ferredoxin was used as the electron donor (173). No inhibition occurs when methyl viologen was the

occur (181-183).

The inactivation of nitrate reductase by ammonia in algae is known to be readily reversible (79,178,184). In C. fusca (178), C. caldarium (79) and Chlamydomonas reinhardi (184) nitrate reductase is reactivated in vivo as soon as ammonia is withdrawn from the medium. The enzyme can also be reactivated in vitro by keeping C. fusca extracts containing the inactivated nitrate reductase at 0°C for several hours (178). In studies with the N. crassa enzyme, however, such a reactivation was not possible (185). In C. reinhardi (184) in vitro-reactivation was achieved by treating extracts containing the inactivated enzyme with ferricyanide or nitrate.

All the available evidence indicated that nitrate reductase activity is not inhibited in vitro by ammonia or amino acids (59,174,175,180). This suggested that inactivation of nitrate reductase in vivo by ammonia is not due to direct inhibition of the enzyme activity. Evidence (12) obtained indicated that ammonia exerts its regulatory effect by uncoupling photosynthetic phosphorylation thereby leading to a rise in the cellular level of the reduced pyridine nucleotides and adenosine diphosphate as a consequence increasing the redox level of the cell (186). Reversible inactivation of nitrate reductase can thus be equally achieved in the light by arsenate, another chemically unrelated uncoupler (186), and in the dark, by stopping aeration of the culture. On the other hand, in vivo-inactivation by arsenate or ammonia does not occur when accumulation of reducing power is hindred, either by speeding

up its reoxidation or by blocking the non-cyclic electron flow of photosynthesis (186).

Interconversion of the active and inactive forms of nitrate reductase could also be achieved *in vitro* by reducing and ~~oxidising~~ oxidising the enzyme with its physiological or artificial substrates, using both crude extracts (184,187) and partially purified preparations (188). Thus, *in vitro* conversion of active *Chlorella* nitrate reductase into its inactive form was found to depend on its reduction by NADH in the presence of ADP, and is prevented by high concentrations of nitrate. The transformation is reversible. Upon reoxidation with ferricyanide, the enzyme becomes active again (189). Nitrate reductase from yeast (70) behaves in a similar way. Thus, the phenomenon seems to be a general property of the enzyme.

Vennesland and her associates (190,191) found that *C. vulgaris* nitrate reductase in the cell-free extracts is largely in an inactive form, which can be extensively activated by nitrate in the presence of phosphate buffer at low pH. Addition of NADH in the absence of nitrate led to a loss of enzyme activity. More recently, the same group has reported that the activation of the inactive form requires an oxidising agent and is inhibited by CO (192). Ferricyanide causes the conversion of inactive to the active form within few minutes even at 0°C, whereas activation by molecular oxygen is slow requiring many hours at room temperature. In *U. maydis*, nitrate reductase is rapidly lost when the organism is transferred to ammonia media (174). The rapidity of ammonia-triggered loss is

suggestive of an active mechanism degrading the enzyme. Since the cycloheximide and actinomycin D, inhibitors of protein and mRNA synthesis, blocked this degradation, it was suggested (174) that a controlling macromolecule induced by ammonia, causes the degradation of nitrate reductase. In studies with N. crassa however, cycloheximide was found to only partially protect nitrate reductase and other related activities from in vivo-inactivation caused by the addition of ammonium tartarate to, or the removal of nitrate from, the cultural medium (185).

In N. crassa, the rapid decline of nitrate assimilating enzymes is caused by the lack of nitrate rather than by the presence of ammonia (140). An active mechanism similar to that proposed for U. maydis nitrate reductase (174) was suggested.

A specific protein, which mediates the in vitro-inactivation of NADH-dependent nitrate reductase, has been reported from the mature roots of maize seedlings (193). The inactivating-enzyme has been purified 460-fold and its molecular weight estimated to be approximately 44,000 (194). The demonstration of the degradation of casein by the inactivating-enzyme suggests that the inactivation of nitrate reductase may be due to its proteolytic degradation.

More recently, Kadam et al. (195) isolated from roots of rice seedlings a heat-labile non-dialyzable factor which inhibits nitrate reductase activity from leaves when NADH or $FMNH_2$ was used as the electron donor. The activity of NVH-nitrate reductase was, however, not affected. In contrast to the NO_3R-inactivating enzyme from maize seedlings (193) the

inhibitor from rice seedlings did not appear to be a general proteolytic enzyme since it had no effect on nitrite reductase and also the inclusion of bovine serum albumin or casein in the incubation mixture did not offer any protection to the inactivation of nitrate reductase. It was suggested that the inhibitory factor present in the roots probably controls NO_3^- assimilation thereby regulating the supply of NO_3^- to the shoots.

Hynes (196) reported the rapid loss of nitrate reductase activity in A. nidulans when the organism was starved for carbon source. The rates of inactivation were similar when brought about by carbon starvation in the presence of the inducer or inducer plus ammonium, or in the absence of the inducer, suggesting that there is only one mechanism of inactivation operating under various conditions.

At present, interconversion of nitrite reductase between an active and an inactive form has only been reported in bacteria using purified enzyme preparations (156,161). The interconversion process seems to be of general metabolic significance, probably related to a redox change of the enzyme protein. Kemp and Atkinson (161) observed that the activity of E. coli NADH-nitrite reductase was enhanced by preincubation with nitrite and decreased by preincubation with NADH. Vega et al. (156) have recently shown that NADH-nitrite reductase from A. chroococcum can be inactivated by preincubation with NADH in the absence of nitrite and that nitrite specifically prevents and reverses such an inactivation. NADPH was also effective in inactivating the A. chroococcum enzyme but the

NADPH-induced inactivation could not be prevented or reversed by nitrite. Furthermore, protection by nitrite against inactivation by NADH is specific, in the sense neither nitrate nor ammonia are effective in this respect (156).

ii) Respiratory Nitrate Reduction

Since the classical experiment of Gayon and Dupetit (197) on the inhibition of dissimilatory reduction of nitrate, the influence of oxygen on the formation and activity of the nitrate and nitrite reducing enzymes in denitrifying bacteria has been a matter of considerable interest (198,199). Oxygen appears to inhibit the formation of enzymes involved in anaerobic respiration (41,200).

The formation and activity of the enzymes catalyzing the reduction of nitrate and nitrite are influenced in several ways: (a) the formation of nitrate and nitrite reductases is repressed by oxygen (41,46).

(b) the function of these reductases is prevented by oxygen, as in the presence of saturating concentrations of oxygen, electron transfer to inorganic electron accepters does not occur, though the reductases are present (41,46).

The mechanism by which oxygen controls the synthesis of anaerobic respiratory enzymes has been the subject of some discussion. Pichinoty (41) suggested that oxygen itself was responsible for the repression of enzymes that appear under anaerobic conditions of growth. Wimpenny and Cole (201) on the contrary, suggested that the redox potential of the growth medium is the controlling factor and that oxygen exerts

its effect by influencing the redox potential. The findings of Showe and DeMoss (9) also revealed that the controlling factor may be the effective intracellular redox potential. This potential is a function not only of the oxidants and reductants in the cell but also its catalytic capabilities for electron flow. Similar conclusions have been drawn by Sinclair and White (202) with cultures of Haemophilus parainfluensae.

The decline in total enzymatic activity immediately after the switch from anaerobic to aerobic conditions and the results of experiments from cell-free preparations show that oxygen inactivates anaerobic nitrate reductase of A. aerogenes both in vivo and in vitro (46) and represses its further formation.

In E. coli, biosynthesis of nitrate reductase is depressed in the absence of nitrate by anoxia (absence of oxygen) alone; but in the presence of nitrate, synthesis is initiated when oxygen tension is significantly lowered even before anoxia is reached (9). It is suggested, therefore, that oxygen functioning as electron acceptor is the repressing agent rather than oxygen per se.

In Bacillus stearothermophilus, the quantity of nitrate reductase that is produced is directly related to the concentration (upto 20 mM) of nitrate and inversely related to the quantity of oxygen in the cultures (101). Aeration of anaerobically grown cells results in the rapid destruction of existing nitrate reductase with or without added amino acids (101).

Nitrite reductase from B. stearothermophilus was also shown to be oxygen-repressible (101).

iii) Influence of light on the activity of nitrate and nitrite reductase in plants

Light plays an important role in the synthesis of nitrate reductase in algae (203) and higher plants (204-206). The activity of nitrate reductase does not increase in leaves exposed to light in CO_2-free atmosphere or in leaves treated with inhibitors of photosynthesis (207). It was suggested that redox changes associated with Hill reaction might be involved in the induction of both nitrate and nitrite reductases (208,209). In the case of tobacco leaves, however, it was reported that the requirement for light could be replaced by providing appropriate amounts of gibberellic acid and kinetin in the dark, suggesting thereby that light per se is not required but acts by supplying these hormones (210,211). It is believed that roots are important centres for the synthesis of hormones which are transported to the shoots (212,213). It has recently been reported that excision of roots depresses the synthesis of nitrate reductase and nitrite reductase in the leaves of rice seedlings and application of gibberellic acid to the excised seedlings restores the level of enzyme synthesis (214). However, even in the presence of gibberellic acid and kinetin, light is still required for the induction of the enzymes. This is contrary to the observation in the tobacco leaves where gibberellic acid and kinetin were reported to induce the synthesis

of nitrate reductase in the dark (210,211). Since the latter experiments were conducted in the dark with young leaves of tobacco plants which were grown for two months in light, the observed synthesis was suggested to be a manifestation of the residual effect of light (214). The residual effect of light in rice seedlings is reported to persist about 12 hr after transfer to the dark (209). The exact mechanism of the influence of light on nitrate and nitrite reductases, however, is yet far from clear.

S E C T I O N VI

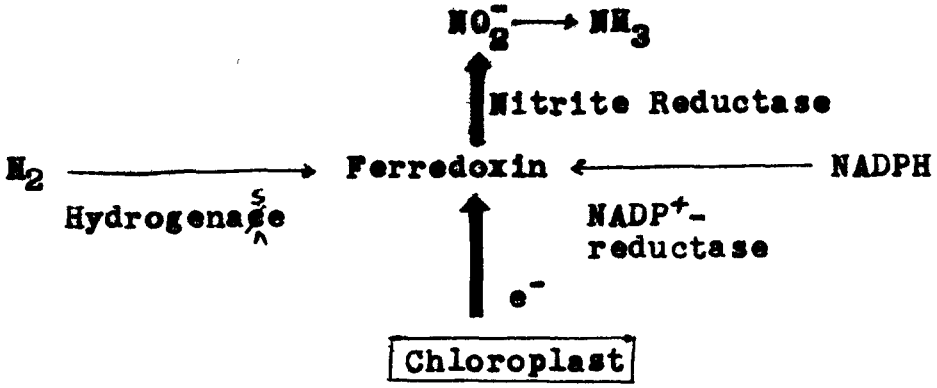
MECHANISM, SEQUENCE OF ELECTRON FLOW AND COFACTORS

1) Algae and Higher Plants

The enzyme involved in the assimilatory reduction of nitrite to ammonia in algae and higher plants has been characterized in recent years and classified as ferredoxin-nitrite reductase (23,71,72). Enzymatic reduction of nitrite was first described by Nason *et al.* (116); soybean leaf extract catalyzed ammonia formation from nitrite in the presence of either NADH or NADPH, and manganese ions. Similarly, in a brief report Vaidyanathan and Street (117) showed NADH-dependent disappearance of nitrite with aqueous extracts of tomato roots, but only 2% of the added nitrite was recovered as ammonia. The studies on the reduction of nitrite by

A. braunii led to the assumption that reduced pyridine nucleotides are generated through hydrogenase (215), and that nitrite reduction was markedly accelerated by exposure to light (216). These studies were confirmed in E. gracilis (131). Both Cresswell (217) and Sanderson and Cocking (120) concluded that reduced pyridine nucleotides did not function as direct electron donors for nitrite reductase. A new insight into nitrite metabolism was provided by the demonstration of a nitrite reductase in cell-free extracts of higher plants capable of reducing nitrite and hydroxylamine to ammonia (118). The use of BVH as electron donor was first reported by Sadana and McElroy (95) for nitrate reduction. Huzisige and Satoh (218) reported a photosynthetic nitrite reductase from spinach leaves but the reaction product was not identified. Nitrite disappearance in the presence of chloroplasts was markedly stimulated in the light. The participation of photosynthetically reduced electron donor in the nitrite reduction was confirmed in a similar grana-enzyme system of tomato (120). With the observation that naturally occurring electron transporting proteins, the ferredoxins, were important not only in the bacteria (219) but also in photosynthetic mechanisms of higher plants (220,221), it became apparent that ferredoxin might also be involved in nitrite reduction. The investigations of a number of groups (121,222-224) revealed that ferredoxin was apparently the physiological electron carrier for the reduction of nitrite to ammonia and could replace viologen dyes as the electron donor. Reduced ferredoxin for the enzymatic reduction

of nitrite can be supplied by light in the presence of grana or in dark by NADPH via $NADP^+$-reductase and hydrogen gas-*Clostridium* hydrogenase system as follows (122)



For each mole of nitrite reduced, 1 mole of ammonia, 1.5 moles of oxygen, and 3 moles of ATP are formed (121,122).

Fungi

Nason *et al.* (116) partially purified from *N. crassa* (and from soybean leaves) a nitrite reductase which catalyzed the reduction of ammonia via hydroxylamine by reduced pyridine nucleotides. The enzyme has a specific FAD requirement as well as a metal component. Nicholas *et al.* (137) further purified *N. crassa* nitrite reductase and concluded that it was NADPH-dependent and contained FAD, Fe, Cu and -SH groups. The enzyme also required Mg^{++} and pyridoxine for maximal activity.

An assimilatory nitrite reductase which utilizes NADPH but no NADH, as electron donor has recently been reported from yeast *T. nitratophila* (70). FAD was a specific requirement for the NADPH-linked activity of nitrite reductase. MVH and

BVH could also be used as electron donors. On the basis of the specific FAD requirement for the enzymatic reduction of nitrite with NADPH and inhibition studies, it was suggested that NADPH-nitrite reductase from T. nitratophila is a metalloflavoprotein.

Bacteria

Relatively k less information is available about assimilatory nitrite reductases from bacteria in contrast to nitrite reductases from plant kingdom (20-22).

Spencer et al. (155) found in extracts of Azotobacter agile a soluble nitrite reductase which also showed hydroxylamine reductase activity. The enzyme utilized reduced nicotinamide nucleotides as electron donors and required added flavin nucleotides for maximal activity. Inhibitor studies indicated that the system had an essential metal component. The product of nitrite reduction was identified as NH_3, whereas that of hydroxylamine was not established.

Extracts of Desulfovibrio desulfuricans reduced nitrite and hydroxylamine to ammonia with either hydrogen gas or pyruvate as hydrogen donor (225,226). E. coli grown in deep-standing cultures with nitrate as the sole nitrogen source has been shown to contain at least three nitrite reductases that catalyze the reduction of nitrite and hydroxylamine to ammonia at the expense of three different reducing agents.

One is specific for NADH, one for NADPH and third, which apparently involves a cytochrome, requires flavin or viologens (161). The NADPH-specific enzyme was shown to function in the intact cell as a sulfite reductase (161).

Vega et al. (156) have recently reported a soluble assimilatory nitrite reductase from A. chroococcum grown anaerobically on nitrate as the nitrogen source. The enzyme utilizes NADH as an electron donor and requires FAD for maximal activity. Inhibitor studies suggested the involvement of thiol groups and a metal component.

ii) Six-electron enzymatic reduction of nitrite to ammonia

It was postulated earlier that reduction of nitrite to ammonia proceeds by a series of two electron transfers, each catalyzed by a different enzyme (20-22). However, working with E. coli nitrite reductase, Lazzarini and Atkinson (160) concluded that the enzyme catalyzed the complete six-electron reduction of nitrite to ammonia with no obligate free intermediates. The relevant findings were: (a) there was no indication of separation of enzymes catalyzing the different steps in the course of moderate degree of purification of the overall system. (b) Possible intermediates at the +1 oxidation level of nitrogen (hyponitrite and nitrous oxide) were not reduced. (c) Although hydroxylamine is reduced, free hydroxylamine is not an obligate intermediate in the reduction of nitrite. (d) There was no evidence for the participation of a dissociable organic cofactor, so that the movement of hydroxylamine from one site to the other in the form of an organic compound seems unlikely. This conclusion was supported by studies of Kemp and Atkinson (161) who showed that E. coli nitrite reductase catalyses the reduction of nitrite as well as hydroxylamine to ammonia and that the Michaelis constant for hydroxylamine was 150-times greater

than that for nitrite. The high Km for hydroxylamine seems to exclude hydroxylamine as an intermediate in nitrite reduction. Similar conclusions were reached with ferredoxin-nitrite reductase from Chlorella, spinach and squash leaves (23, 72). Results reported recently by Vega et al. (156) corroborate this view and show that in A. chroococcum, nitrite is stoichiometrically reduced to ammonia without the formation of hydroxylamine as a free intermediate.

Since ferredoxin is a single electron donor, a nitrite reduction to ammonia might proceed by a series of one-electron stages; such a scheme was proposed by Fewson and Nicholas (227). However, if such intermediates do occur it appears unlikely that they are released in the free form. Hewitt et al. (127) and Cresswell et al. (171) suggested a tentative hypothetical scheme, based on a hemiacetal structure, for the reduction of nitrite and hydroxylamine by a single protein (Fig. 1). The scheme would be compatible with the absence of free intermediates. However, as the scheme is based on a sequence of two-electron steps it would need further modifications to accommodate the specific requirement for a single electron donor (either ferredoxin or benzyl viologen), as suggested by the authors (127).

Other six electron reduction reactions which occupy crucial position in metabolism and which involve only a single enzyme are those catalyzed by sulfite reductase, $[SO_3^{2-} + 6(H^{\cdot}) \rightarrow S^{2-} + 3\ H_2O]$ and nitrogenase $[N_2 + 6(H^{\cdot}) + 2\ H^+ \rightarrow 2\ NH_4^+]$. Like nitrite reductase, assimilatory sulfite reductases have been obtained in a

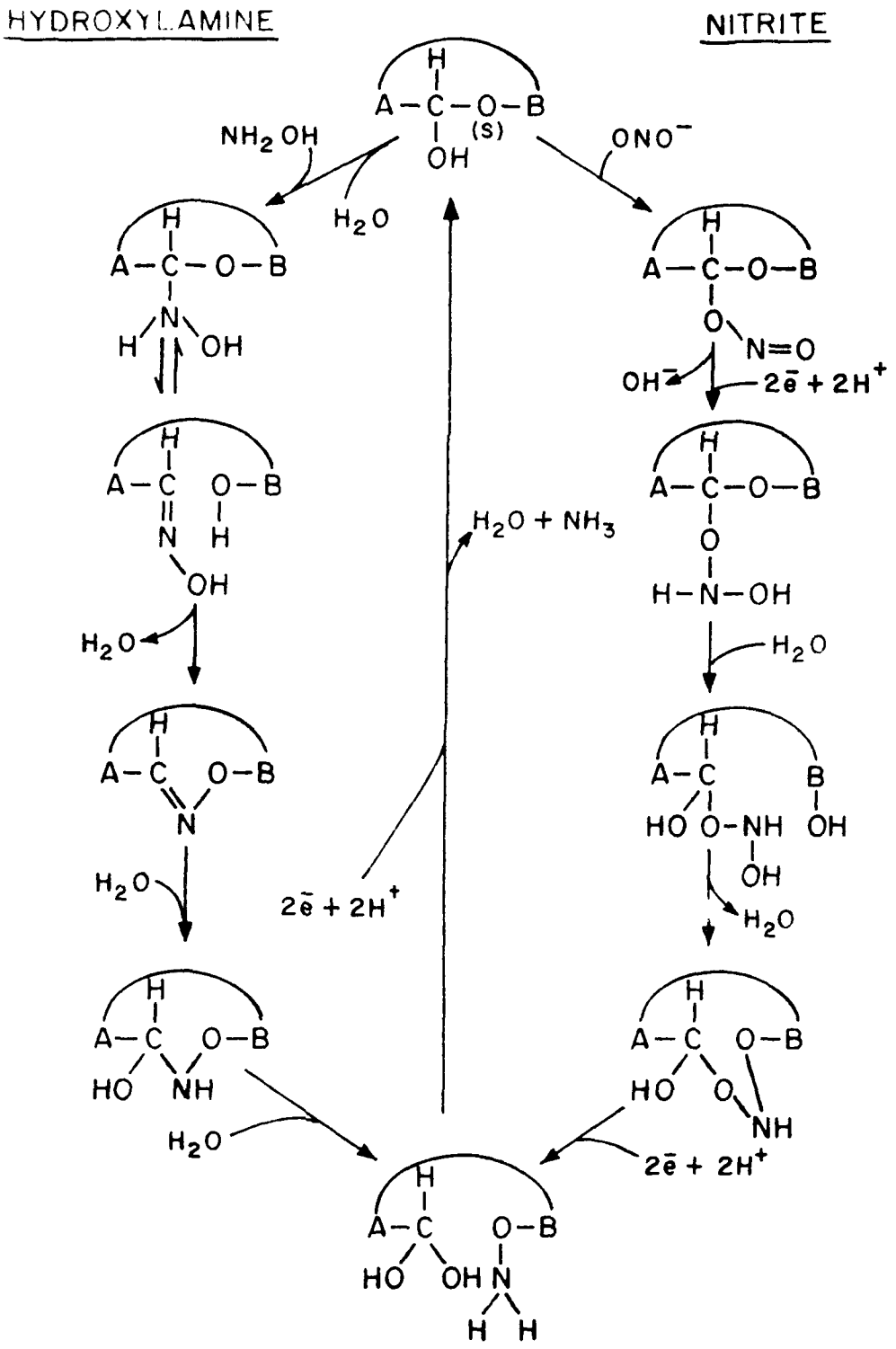


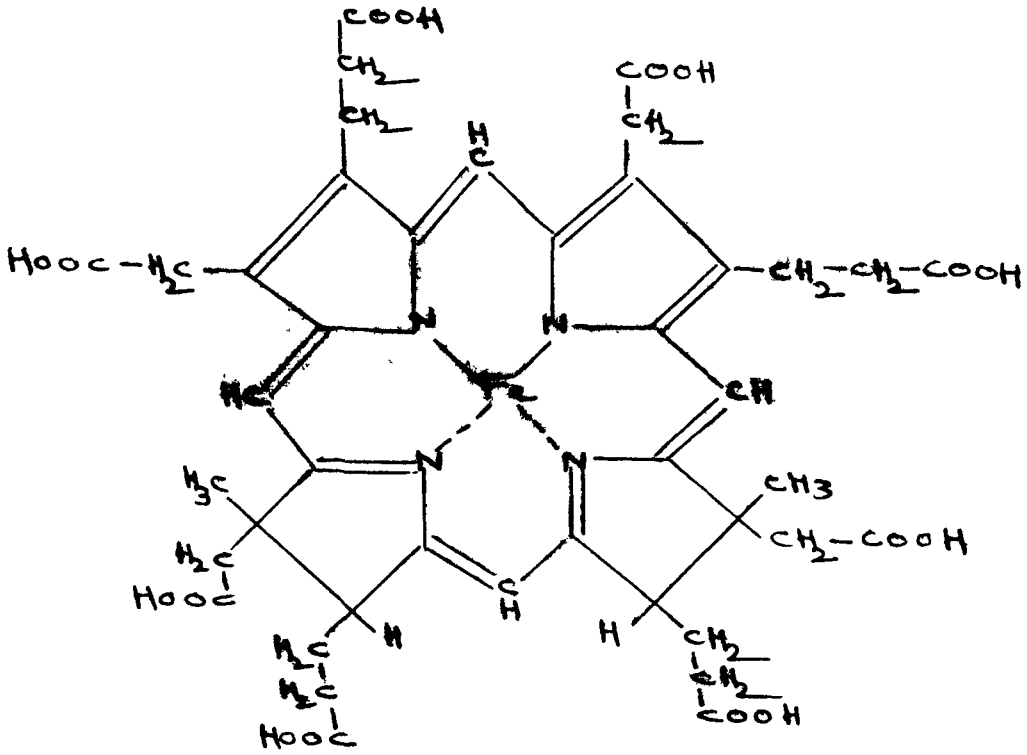
FIG. 1 TENTATIVE SCHEME FOR REDUCTION OF NITRITE OR HYDROXYLAMINE BY SAME ENZYME SYSTEM PRODUCING NO FREE INTERMEDIATES

homogeneous state which catalyze the six electron reduction of sulfite to sulfide without the formation of free inorganic sulfur-containing intermediates (228,229).

Although in plants, sulfite and nitrite reductions are catalyzed by distinct enzymes (230,231) it is interesting to note that in bacteria and fungi, sulfite reductases of both assimilatory (160,161,229,232,233) and dissimilatory (234) types are capable of catalyzing the reduction of nitrite. With E. coli sulfite reductase the product of nitrite reduction by NADPH was identified as ammonia (160). The possibility that some structural features may be common to both sulfite and nitrite reductions in nature was supported by the studies of Zumft (135) who demonstrated striking similarity between nitrite reductases (from Chlorella and spinach) and sulfite reductases (from spinach, yeast and E. coli) suggesting that the two enzymes might share a common heme chromophore. The heme chromophore has recently been isolated from E. coli sulfite reductase and identified as a new type of heme prosthetic group, an octacarboxylate iron-tetrahydroporphyrin of the isobacteriochlorin type which has been given the name "siroheme" (235,236).

Seigel and his colleagues have reported a detailed study of E. coli sulfite reductase. The enzyme is complex hemo-flavoprotein of molecular weight 670,000. It contains per molecule (22) 4 FAD, 4 FMN, 20 to 21 atoms of iron, approximately 16 atoms of acid-labile sulfide, and 3 to 4 molecules of siroheme (235,236). The FAD prosthetic group is bound much more

tightly than the FMN (238).



Postulated structural formula for
the siroheme prosthetic group

The enzyme is composed of only two different types of polypeptide chains, termed ∝ and β, which may be dissociated by urea treatment and separated on DEAE-cellulose chromatography (239). The ∝-chains bind the FMN and FAD prosthetic groups, while the β-chain bind the iron, sulfur and siroheme prosthetic groups. The subunit structure appears to be $\alpha_8\beta_4$. The flavoprotein (∝-chains) can catalyze the NADPH-dependent reduction of a variety of artificial electron acceptors, including cytochrome c, but not that of the natural enzyme acceptor, sulfite. The hemoprotein (β-chains), on the other hand, can catalyze the reduction of sulfite with artificial electron donor, MVH, but

not with the natural electron donor NADPH. The isolated flaveprotein and hemoprotein can combine to reconstitute the complete electron transfer sequence with NADPH-sulfite reductase activity (239).

The FAD prosthetic group of sulfite reduction appears to serve as the sole "entry part" for electrons from NADPH (238). The FMN prosthetic group is required for transfer of electrons from NADPH via the FAD, either to enzyme-bound siroheme and thence to sulfite, or to the exogenous electron acceptor, cytochrome c. The majority of electrons which are transferred from NADPH to the 'diaphorase'-type acceptors 2,4-dichlorophenol indophenol, ferricyanide, or menadione by sulfite reductase pass through a FMN-dependent pathway (238). Thus, NADPH reacts with the flavins, while sulfite interacts with the siroheme component. The patterns of interaction of the enzyme with a variety of electron donors, acceptors and inhibitors indicated the following minimum sequence of electron transfer:

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{FMN} \rightarrow \text{heme} \rightarrow SO_3^{--} \text{ or } NO_2^-$$

Siroheme was shown to serve as the prosthetic group for sulfite reductases associated with sulfate respiration as well as sulfate assimilation (236,240). More recently, Murphy et al. (241) reported that the heme-like prosthetic group of spinach ferredoxin-nitrite reductase is identical in its spectral and chromatographic properties to the siroheme prosthetic group of E. coli sulfite reductase. The presence of this new type iron-porphyrin, siroheme, in the assimilatory

sulfite reductase of E. coli and spinach ferredoxin nitrite reductase suggests that both types of "multi-electron reduction processes (sulphite to sulfide and nitrite to ammonia) may share common mechanistic features. This possibility is strengthened by the fact that many highly purified sulfite reductases have been shown to possess nitrite reductase activity. Some of this reactivity, it has been suggested (241), may be ascribed to the siroheme. The association of siroheme with the reduction processes involved in the metabolism of two of the major elements of the biosphere, nitrogen and sulfur, suggests that this novel heme may have played a key role in the evolution of redox metabolism.

Although all adequately studied sulfite redactases contain siroheme as prosthetic group (236,240), this generalization cannot be made for nitrite reductases. Prakash and Sadana (170) have reported from this laboratory that A. fischeri nitrite reductase that catalyzes the reduction of nitrite to ammonia as part of the process of nitrate respiration contains a heme c-type prosthetic group as its sole iron-containing moiety. This is incompatible with the presence of siroheme in the A. fischeri nitrite reductase. Enzymes involved in the dissimilation of nitrite to gaseous nitrogen (NO, N_2O, N_2 etc.) do not have the siroheme prosthetic group.

iii) Denitrifying organisms

In the past, lack of precise analytical techniques slowed the study of stepwise reduction of nitrite in denitrification, but introduction of gas chromatographic methods

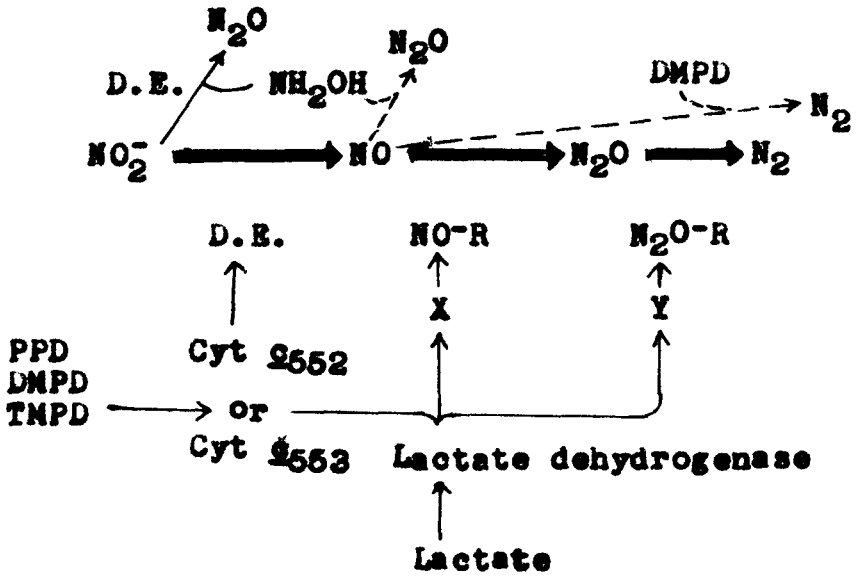
(149,242) has increased the pace of research. Thus several workers determined: (1) that nitric oxide is a specific product of nitrite reduction, (ii) that nitrous oxide results from nitric oxide reduction, and (iii) that nitrous oxide is the terminal denitrification product of several bacterial strains (10,149,243,244).

The following section will deal in brief with various schemes of dissimilatory nitrite reduction in denitrifying bacteria.

Pseudomonas denitrificans

The copper-containing nitrite reductase from P. denitrificans was obtained in an ultracentrifugally homogeneous state (143). The enzyme catalyzed the reduction of nitrite to nitrous oxide using hydroxylamine as an electron donor. Nitric oxide was identified as the reduction product of nitrite with bacterial cytochrome c552 as a specific hydrogen carrier and tetramethyl p-phenylenediamine (TEMPD) as a hydrogen donor (245) When dimethyl-p-phenylenediamine (DMPD) or p-phenylenediamine (PPD) were added to this system, nitrogen was evolved instead of nitric oxide (the nitric oxide produced reacts with the phenylamino or imino groups present to give nitrogen). This observation suggested that nitric oxide was an intermediate of the denitrification reaction. Miyata and Mori (246) working with various reconstituted reaction systems, demonstrated that nitric oxide was the only product of nitrite reduction catalyzed by the denitrifying enzyme(NO_2R). Matsubara (247) has recently shown

that the mechanism of gas production from DMPD and nitrite and from NH_2OH and NO_2^- is different from the normal denitrifying mechanism using a respiratory substrate, lactate, as the sole reducing agent. The following scheme, which is a modification of earlier schemes (244), represents a possible mechanism of the nitrite reduction to N_2 in P. denitrificans (247):



➡ Normal denitrifying process

--→ Non-enzymatic reactions

⟶ Other enzymatic reaction, or electron transport system

D.E., Denitrifying enzyme (nitrite reductase); NO.R., NO reductase; N_2OR, N_2O reductase; X,Y, unknown compounds of electron transport system

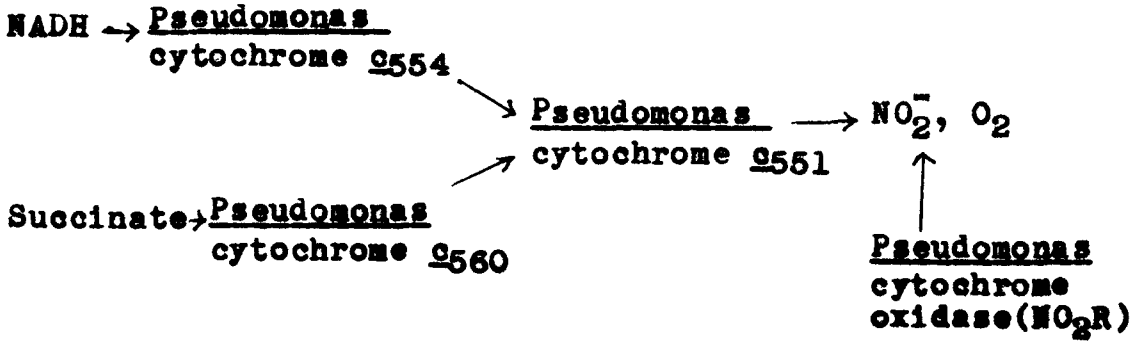
Pseudomonas aeruginosa

A 600-fold pure preparation of nitrite reductase from P. aeruginosa was reported to contain FAD, Fe and Cu as components and showed a cytochrome c-type spectrum (145). The enzyme reduced nitrite to nitric oxide when either reduced

flavins, reduced pyocyanine or reduced methylene blue was the electron donor. FAD was required for maximal activity. NADH, NADPH and reduced cytochrome c were ineffective as electron donors. The following scheme was proposed:

$$\begin{array}{lll} & & Cu^{1+} \rightleftharpoons Cu^{2+} \\ PyH_2 & \longrightarrow FAD \longrightarrow & \text{Cu. Protein} \longrightarrow NO_2^- \end{array}$$

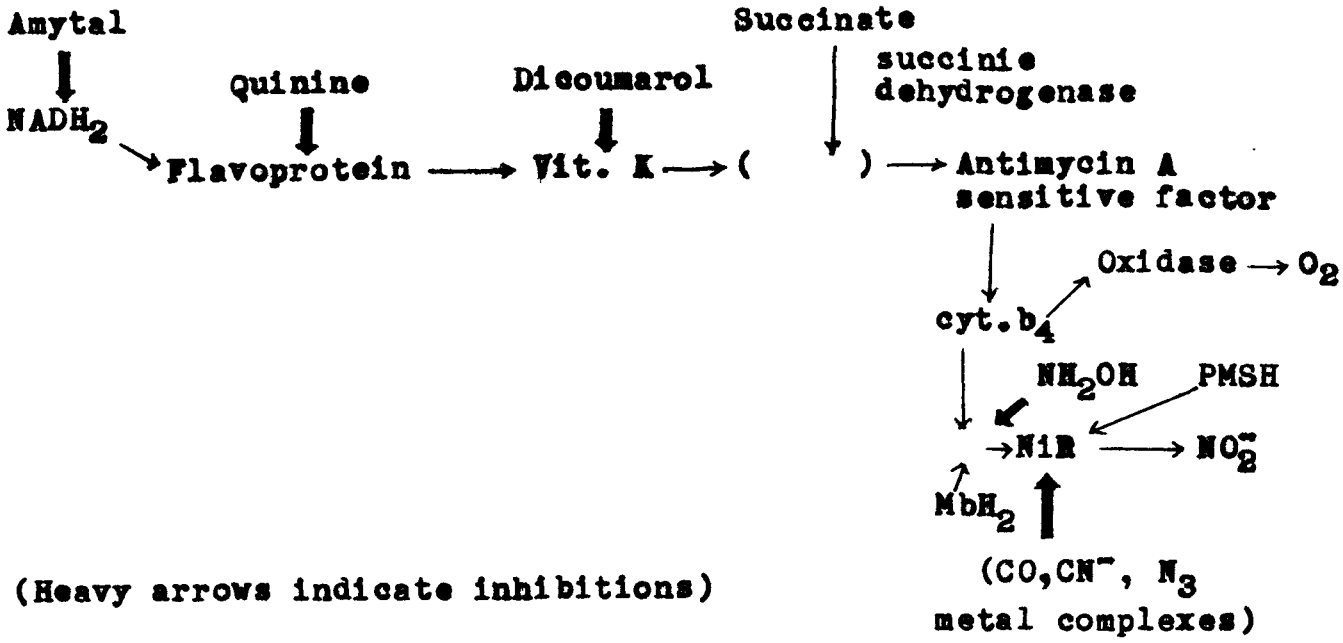
Yamanaka et al. (147) obtained a purified preparation of the enzyme which reduced nitrite to nitric oxide with Pseudomonas cytochrome c_{551} as the electron donor. The enzyme contained two types of hemes, heme a_2 (heme d) and heme-c and also showed strong cytochrome oxidase activity (248). The following scheme was suggested for the reduction of nitrite in P. aeruginosa.



Micrococcus denitrificans

In Micrococcus sp. nitrite is totally reduced to nitrogen with one mole of nitrogen being formed for every two moles of nitrite reduced. A NADH-dependent nitrite reductase was reported which was stimulated by added FAD and menadione and inhibited by amytal, quinine, dicoumarol, antimycin A and CO (148). Besides reduced dyes which served as direct electron donors,

a cytochrome b_4 seemed to function as an electron carrier. The following scheme was suggested:



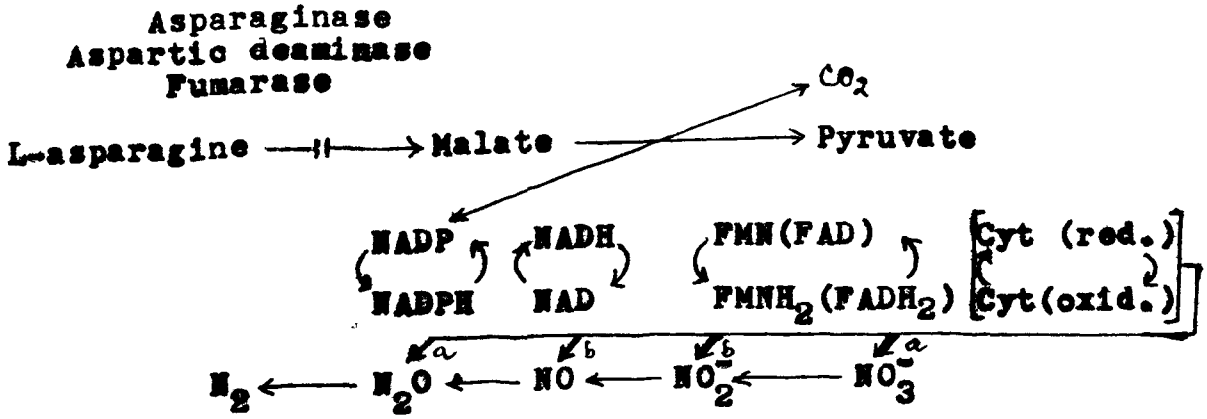
(Heavy arrows indicate inhibitions)

A cytochrome-containing protoheme was suggested to be participating at the step shown with blank parenthesis. The Micrococcus nitrite denitrifying system was subsequently separated into two, particulate and soluble, protein fractions, both were necessary for denitrification. Soluble activity was stimulated by Cu^+ and Cu^{++}, whereas the particulate activity was enhanced by Fe^{++} and Fe^{+++}. Unlike P. denitrificans, nitrite reduction in M. denitrificans was reported to be coupled with phosphorylation, though to a lesser extent than that for nitrate (38). Like several denitrifying bacteria which require cytochrome c as an electron carrier, nitrite reductase of M. denitrificans is linked to the electron transfer chain at a site between cytochrome c and O_2 (249). A 99% pure preparation

of nitrite reductase was reported from M. denitrificans (110). Nitrite reductase from M. denitrificans like P. aeruginosa enzyme was a cytochrome containing two hemes, heme c and heme d and exhibited cytochrome oxidase activity (110).

Pseudomonas perfectomarinus

In P. perfectomarinus which requires asparagine for denitrifying growth, the reduction of nitrite to nitrogen consists of three identifiable steps (113), each involving a different enzyme system. From crude extracts of P. perfectomarinus, grown anaerobically on nitrate, nitrite or nitrous oxide, separate complex fractions were obtained that utilized NADH as electron donor for the reduction of (i) nitrite to nitric oxide (ii) nitric oxide to nitrous oxide, and (iii) nitrous oxide to nitrogen. Each of these fractions reduced only one of the nitrogenous oxides (10). Nitrite and nitric oxide reductases were found soluble and were partially purified, whereas nitrous oxide reductase remained particle-bound (113). Electron flow was initiated by $NADP^+$-linked malate dehydrogenase, connected in turn by transhydrogenase to NAD^+, and on to free flavin reduction. The following scheme was suggested for denitrification in P. perfectomarinus (18):



a Membrane associated

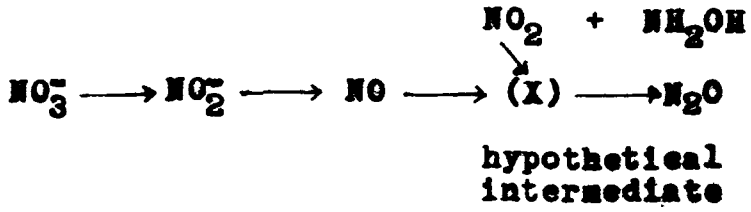
b Soluble

Electron flow for

denitrification in P. perfectomarinus

Other Denitrifiers

The end product of dissimilatory nitrate and nitrite reductions in Corynebacterium nephridii is nitrous oxide. Nitric oxide was detected during the reduction of nitrite to nitrous oxide (149). Like P. denitrificans, C. nephridii can also convert hydroxylamine and nitrite to nitrous oxide in the presence of lactate as electron donor. A tentative scheme suggested for dissimilatory nitrite reduction in C. nephridii is as under:



Iwasaki and Matsubara (150) obtained from *A. cycloclastes* a highly purified preparation of a copper-containing nitrite reductase which catalyzed the production of nitric oxide from nitrite using ascorbate-phenazine-methosulfate as the electron donor. Like *Pseudomonas* (163) and *Corynebacterium* (149) enzymes, *A. cycloclastes* enzyme can also catalyze nitrous oxide production from nitrite and hydroxylamine.

A two-heme cytochrome acting as a nitrite reductase in dissimilatory nitrite reduction has been reported from *A. faecalis* (112). The enzyme catalyzes the formation of NO from NO_2^- in the presence of ascorbate and phenazine-methosulfate. The authors suggested that cytochrome *c d* of *A. faecalis* may function as the two-heme nitrite reductases from *P. aeruginosa* and *M. denitrificans*.

SECTION VII

PRESENT INVESTIGATION

It is evident from the literature reviewed in this chapter that nitrite reductases have mostly been studied in either crude preparations or in partially purified form. Studies with relatively pure enzyme preparations have, however, been reported in the case of *P. aeruginosa* (111), *M. denitrificans* (110), *C. pepo* (165), *A. faecalis* (112), and *A. cycloclastes* (150). The enzymes obtained from *C. fusca* (135), and spinach (136) are homogeneous in disc gel electrophoresis. It is also evident from the literature reviewed here that little information is available on the subunit structure of nitrite reductases. The investigations made till now indicate that nitrite reductases from different organisms have widely different properties and different reaction mechanisms. The work presented in this thesis includes the following investigations:

1. Purification of the enzyme to a state which is homogeneous in the ultracentrifuge and disc gel electrophoresis and study of some of its physical properties.
2. Studies on the subunit structure of the enzyme.
3. Determination of the amino acid composition and some of the hydrodynamic parameters of the enzyme.
4. Studies on sulfhydryl and disulfide groups and the involvement, if any, of -SH groups in the enzyme activity.

5. Studies on the reversible inactivation of the enzyme by urea, guanidine HCl, sodium dodecyl sulfate and acid pH.

These studies, it was hoped would throw some light on the structure of the enzyme molecule and may also form the basis for future studies on the mechanism of the six-electron reduction of nitrite to ammonia by A. fischeri nitrite reductase.

Chapter 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

All the chemicals used in the media for the growth of *Achromobacter fisheri* were of analytical grade. Bacto peptone and agar (Difco) were obtained from Difco Laboratories, U.S.A. and beef extract from Oxoid, England. Ferric chloride and glycerol were supplied by B.D.H., England.

Crystalline bovine serum albumin, crystalline ovalbumin, catalase, yeast alcohol dehydrogenase, deoxyribonuclease I, lysozyme (three times crystallized), dansyl chloride, dansyl amino acids kit, p-hydroxymercuribenzoate, p-chloromercuribenzene sulfonic acid, 5,5'-dithiobis (2-nitrobenzoic acid), ~~5,5'-dithiobis (2-nitrobenzoic acid)~~, reduced glutathione, protamine sulfate (Salmine) were obtained from Sigma Chemical Company, U.S.A. Acrylamide, N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylethylenediamine were the products of Eastman Organic Chemicals, U.S.A. Benzyl viologen, methyl viologen, tyrosine, tryptophan, ethylenediaminetetracetic acid and p-dimethylaminobenzaldehyde were purchased from British Drug Houses Ltd., England. Sephadex G-200, and Blue Dextran 2000 were obtained from Pharmacia, Sweden and Bio-gel P-150 from Bio-Rad Laboratories, U.S.A.

The following chemicals were purchased from the suppliers indicated: 1-nitroso-2-naphthol (Hopkins and Williams); sodium borohydride (Koch and Light Laboratories Ltd.); 2-mercapto-ethanol (Fluka, Switzerland); sodium dodecyl sulfate (HICO

Products Pvt.Ltd., Bombay); sulfosalicyclic acid (Riedel, Germany).

All other chemicals were of analytical grade and were obtained from commercial sources. Prior to use, p-hydroxymercuribenzoate was crystallized by the procedure of Boyer (250). Sodium dodecyl sulfate was crystallized twice from ethanol before use.

Guanidine hydrochloride was prepared from guanidine carbonate (Analar, B.D.H.) according to the procedure of Kawahara and Tanford (251). The carbonate was recrystallized from aqueous solution by the addition of ethanol at 4°C, the crystals dried in vacuum and mixed with water to make a paste and converted to hydrochloride by the addition of cold concentrated HCl. The resulting solution was adjusted to pH 5.4, filtered and concentrated in vacuo below 40°C and the residual mass recrystallized from methanol. The crystals were stored dry in a vacuum desiccator over P_2O_5 and solutions prepared and used fresh.

The analytical grade urea (B.D.H.) was recrystallized from aqueous ethanol and the crystals stored dry over P_2O_5 in vacuum. Solutions were made fresh before use.

Hydroxylapatite gel was prepared according to the procedure of Tiselius, Hjerten and Levin (252). The gel was equilibrated with 0.0001 M potassium phosphate buffer (pH 6.8). Nitrogen gas was obtained from Indian Oxygen Ltd., Bombay and was made O_2-free by passing over heated copper at 700-800°C and then through methylene blue solution reduced by hydrogen and

palladised asbestos (Danpha Chemicals, India).

Methods

Organism: The salt-water luminous bacterium Achromobacter fischeri used in the present investigation was kindly supplied by Dr. W.D. McElroy (McCollum-Pratt Institute, Johns Hopkins University, Baltimore, U.S.A.). The culture appeared as Gram-negative rods, sometimes slightly curved.

Maintenance and propagation of culture: A. fischeri was propagated on nutrient agar slant of the following composition:

Peptone	0.5 g
Sodium chloride	3.0 g
Beef extract	0.3 g
Glycerol	0.3 ml
Agar	2.0 g
Calcium carbonate	0.3 g
Distilled water to make	100 ml

The first four constituents were dissolved in water and the pH was adjusted to 7.4 with 4N KOH. The final volume was then made to 100 ml. Agar and calcium carbonate were then added and the mixture steamed for one hour. For preparation of slants, 7-8 ml aliquots were distributed into 19 x 150 mm Pyrex test tubes and autoclaved at 15 psi (120°C)for 20 min. The tubes were shaken while hot in order to distribute calcium carbonate uniformly, immediately slanted and allowed to solidify. These slants were inoculated from the stock culture

and incubated at 28°C for 24 hrs.

The organism was maintained at 4°C and subcultured routinely every month.

Basal liquid medium for growth: To obtain large amounts of cells, the organism was grown in the folbwing basal liquid medium:

Sodium chloride, (NaCl)	30 g.
Ammonium phosphate, $(NH_4)_2HPO_4$	0.5 g
Potassium dihydrogen phosphate, (KH_2PO_4)	2.1 g
Disodium hydrogen phosphate, $(Na_2HPO_4 . 12H_2O)$	7.05 g
Magnesium sulfate ($MgSO_4$)	0.1 g
Ferric chloride ($FeCl_3.6H_2O$)	0.01 g
Glycerol	3 ml
Peptone	10 g.
Distilled water to make one liter	
pH was adjusted to 7.4 by 4N KOH	

All the constituents were dissolved separately, then mixed and made to the final volume. The media were autoclaved at 15 psi for 20 min. When nitrite was required to be incorporated this alone was sterilised by passing through a Seits filter.

Growth conditions and collection of cells: Inocula from 24 hr cultures grown on agar slopes were transferred into 500-ml conical flasks containing 100 ml liquid medium and grown for 20 hr at 28°C on a rotary shaker, 210 rpm. The organism was subcultured through two transfers in liquid media (without nitrate) under aerobic conditions. The inoculum was then transferred

to 15 liters of the basal liquid medium in glass carboys containing 0.1% potassium nitrate. Antifoam (0.2 to 0.3 ml, Alkaterge C, Commercial Solvents Corporation, U.S.A.; one part antifoam mixed with four parts of liquid paraffin) was added to each carboy in order to prevent excess frothing. The culture was kept at 28 to 30°C and purified air was continuously forced through the cultures from sintered glass units at 550 ml/min. After growing for 18-20 hr, the cells were harvested in a refrigerated Sharples centrifuge (2,000 rpm) at a flow rate of about 10 liters per hour. The bacteria were washed free of nitrite by suspending in 3% sodium chloride and centrifuging. The cells were stored as a paste at 15°C until used.

Definition of unit of activity and specific activity:

The unit of nitrite reductase activity is defined as the amount of enzyme required to cause disappearance of 1 μmole of nitrite in 10 minutes at 30°C and pH 7.5 using reduced benzyl viologen as the electron donor under the experimental conditions given in the text. The specific activity of the enzyme is defined as the activity per mg of protein.

Estimation of nitrite reductase activity:

Nitrite reductase was measured in Thunberg tubes after evacuation and refilling with O_2-free nitrogen with chemically reduced benzyl viologen as electron donor. The rate of reduction was measured by determining the decrease of nitrite concentration in the reaction mixture by the diazo-coupling procedure of Snell and Snell (253). The details of the assay

procedure are as follows.

The incubation mixture contained, in a final volume of 1.5 ml, 200 μmoles of potassium phosphate (pH 7.5), 0.67 μmoles of $NaNO_2$ and enzyme protein. 0.5 ml of benzyl viologen (10 mg/ml in water) and 1 ml of freshly prepared dithionite (1 mg/ml) in 0.2 M potassium phosphate (pH 7.5) were placed in the side arm of the Thunberg tube and the tubes were evacuated immediately. The reaction was started by addition of reduced benzyl viologen. The final pH of the reaction mixture was 7.5. After 4-6 min of incubation at room temperature, the reaction was terminated by opening the Thunberg tubes and shaking for few seconds to oxidize all the reduced benzyl viologen. To 1 ml of reaction mixture was then added 1 ml of sulfanilamide reagent (1% w/v in 1 M HCl) followed by 1 ml of N-(1-naphthyl)-ethylenediamine dihydrochloride (0.02% w/v in water). The resulting red color was read at 540 nm after 10 minute after making the volume to 9.5 ml. The amount of enzyme used was adjusted so that the nitrite utilized was between 0.2 - 0.3 μmolee. An optical density of 0.5 for 10 mm light path was taken as equivalent to 0.1 μmole of nitrite reduced. A blank with all the assay constituents except enzyme was always run.

Estimation of catalase activity: Catalase was assayed according to Beers and Sizer (254). 2.9 ml of buffered solution of hydrogen peroxide (0.2 ml of 30% H_2O_2 in 50 ml of 0.05 M potassium phosphate buffer, pH 7.0) was taken in 3 ml capacity silica cuvette. 0.1 ml of enzyme sample was then added to the substrate solution and the decrease in optical density per 1-2 min,

used as the standard.

(b) Method of Lowry et al.:

Protein determinations in the subsequent purification steps were carried out with the Folin-Ciocalteu reagent as described by Lowry et al. (257). Crystalline bovine serum albumin was used as the standard and the final solution was read at 750 nm, the absorption peak. Samples free of ammonium sulfate and Tris and containing only low concentrations of phosphate were used to avoid interference from these substances.

(c) Optical method:

Protein determination by the optical method of Warburg and Christian (258) was done by using the following empirical equation (259) to correct for light absorption due to nucleic acids, the light path being 10 mm:

$$4/7 \left[2.3\ (O.D._{280nm} - O.D._{340nm}) - (O.D._{260nm} - O.D._{340nm})\right] = \text{mg protein per ml}$$

This method was used, although it was somewhat inaccurate, to obtain rapid comparative estimates of protein content. The concentrations of serum albumin and mammalian cytochrome c in solutions were calculated from their extinction coefficients at 280 nm (260) and 550 nm (261) respectively.

(d) Micro-Kjeldahl method:

This method was used to determine the protein of electrophoretically homogeneous sample of enzyme to compare the final specific activities obtained by this method and those based on protein determinations by optical and Lowry's methods. The protein was calculated from total nitrogen on the assumption

that the protein contained 16% N. 1 - 1.5 mg enzyme protein, dialyzed against distilled water, was digested with 2 ml of conc. H_2SO_4 and about 1 gm of digestion mixture of composition, 5 mg $CuSO_4.5H_2O$, 0.5 mg powdered selenium and 250 mg $KHSO_4$ (262), until completely clear. The ammonia liberated from the digest by steam distillation under alkaline conditions was absorbed in N/70 H_2SO_4 and nitrogen was calculated by titrating against standard NaOH solution. The accuracy of the method was judged by using standard ammonium sulfate solution. For comparison, protein estimations on standard solution of cytochrome c and bovine serum albumin were also carried out by this method.

Ammonium sulfate fractionation:

Ammonium sulfate saturation refer to 0°C and the quantity required for changing the degree of saturation was calculated according to Jagannathan et al.(259) from the following equations:

$$\text{For solid ammonium sulfate: } X = \frac{50(S_2-S_1)}{1-0.28\ S_2}$$

$$\text{For saturated ammonium sulfate: } Y = \frac{100(S_2-S_1)}{1-S_2}$$

where X equals gm of solid ammonium sulfate to be added to 100 ml of solutions of saturation S_1 in order to change it to saturation S_2, and Y equals ml of saturated solution to be added to 100 ml of solution to change its saturation from S_1 to S_2, S_1 and S_2 being expressed in fractions of saturation at 0°C. Ammonium sulfate, solid or solution, was added slowly

with gentle stirring to avoid frothing and the liquid was allowed to stand for 30-40 min, then centrifuged at 4,000 x g for 45 min.

Ultracentrifugation:

The ultracentrifugal studies were carried out in the Beckman Spinco model E ultracentrifuge equipped with a phase plate-schlieren optics and a rotor temperature indicator and control device capable of maintaining a constant temperature during the run. All the determinations in aqueous system were carried out at 3-8°C, and in denaturing systems at 20-25°C, using red-sensitive I-N spectroscopic plates (Kodak) and a 660 nm red filter for recording the sedimentation profile of the pink colored protein. A counter balance with the usual reference holes was used to provide reference points for determining radical distances from the axis of rotation.

Homogeneity and sedimentation coefficient:

Homogeneity and $s_{20,w}$ were routinely determined from sedimentation velocity runs using a 4° sector, 12 mm standard cell at a speed of 59,780 rpm. Some of the runs were also carried out in a Beckman valve-type synthetic boundary cell of 12 mm thickness and 4° sector. Photographs taken at different time intervals were read either on a Hilger (L-60) or a Gaertner (model M 2060) microcomparator. Correction for the stretching of the analytical rotor (0.02 cm) was determined according to the method of Kegeles and Gutter (263). Sedimentation coefficient was calculated in the usual manner from the plots

of the logarithm of distance of sedimenting boundary from the axis of rotation versus time (264). The sedimentation coefficients (s_{obs}) were normalized to water at 20°C ($s_{20,w}$) after making density and viscosity corrections (264). A value of 0.73 ml/g calculated from amino acid composition as described in text was used after correction for the temperature.

Molecular weight:

Molecular weight determinations were made by the approach-to-equilibrium method of Archibald (265) as described by Schachman (264) in a synthetic boundary cell. The phase plate was used at an angle of 80° and the approximate speed of the centrifugation for linear extrapolation of the gradient curve was calculated according to Labar (266). The protein solution was dialyzed overnight at 0-4°C against 100 volumes of 0.05 M phosphate buffer (pH 6.8) with two changes and then spun at 10,000 x g for 15 min before filling in the cell. 0.6 ml solution (0.5 - 1.0% protein) was used directly in the sector of the synthetic boundary cell with the cup empty for determination of changes in concentration. Only readings at the meniscus were taken. Initial protein concentrations were determined by layering the solvent buffer system (0.2 ml) from the cup at a speed of about 8,000-10,000 rpm over the protein solution (0.4 ml) and immediately adjusting rotor speed to that used in the corresponding run for determining concentration depletion at the meniscus. Photographic plates were read at 0.1 mm intervals either on a Hilger L-50 two-way measuring micrometer with a sensitivity of one micron or a

model M 2060 Gaertner microcomparator. Areas were determined by trapezoidal analysis.

For determining the molecular weight in denaturing systems, the enzyme was precipitated with solid ammonium sulfate and the protein precipitate dissolved in approrpriate denaturing buffer system. The enzyme was dialyzed against the denaturing buffer for 70-80 hr with at least four changes of the buffer. The densities and viscosities for guanidine HCl and urea solutions were taken from tables of Kawahara and Tanford (251).

As a check on the accuracy of the method, the molecular weight of crystalline bovine plasma albumin was determined. Consistent values were obtained and were on the average 68,000 in both dilute aqueous buffer solution and in 8M urea. This is in good agreement with the reported molecular weight values in the literature (267,268). The method of Archibald was preferred over that of Yphantis (269) because of the difficulty in obtaining a stabilized supply of current over a long period of time.

Gel filtration studies:

A column (1.6 x 55 cm) of Sephadex G-200 (40-120 μ) or Bio-Gel P-150 was equilibrated at 4°C with 50 mM potassium phosphate buffer, pH 6.8. Hydrated gel and buffer were routinely deaerated under vacuum prior to use. When the bed had settled to a constant height the sample solution (0.5 - 1.0 ml) containing 10% sucrose was carefully layered under the buffer solution above the gel. Eluate fractions of 1 ml were collected at a flow rate of 10-12 ml/hr and assayed for protein and/or

enzymic activity. Dextran Blue 2000 (Pharmacia) was used to determine the void volume (V_o) and phenylalanine to measure the inner volume (V_i). The total volume (V_t) was determined directly with water. The elution volume (V_e) of a given solute zone was taken in all cases as the effluent peak position of the solute. The column was calibrated with proteins of known molecular weights (270) or Stokes' radii (271). Gel filtration data are presented in terms of V_e/V_o, K_d and K_{av}, the parameters involved in several mathematical correlations of elution volume with Stokes' radius and molecular weight (271-273). The parameters, K_d and K_{av} are calculated as defined by the following equations (274):

$$K_d = \frac{V_e - V_o}{V_i} = \frac{V_e - V_o}{V_t - V_g - V_o}$$

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e, V_o, V_t and V_i have the same meaning as described above. V_g, the volume occupied by the gel grains is estimated from the following relation

$$V_g = V_t/B.d$$

where B = bed volume per gram of dry Sephadex G-200 (approx 35 ml/g) and d is the density of dry Sephadex G-200 (1.65 g/ml) (273). For the columns used in the present work $V_g = 1.73$ and $K_{av} = 0.97\ K_d$.

Polyacrylamide gel electrophoresis:

Analytical disc gel electrophoresis was performed according to Davis (275) using 7.5% acrylamide gel polymerized with 0.07% persulfate. The discontinuous buffer system of Davis was used in which separation gel contains a Tris-HCl buffer of pH 8.9. Sample and stacking gels were omitted. After 2 hr of preliminary electrophoresis to eliminate persulfate ions, 50-200 μl sample (made dense with 20% sucrose) was applied through the upper buffer onto the surface of the gels. Electrophoresis was carried out in the cold room (4°C) at 3 mA per tube for 3-4 hr until the dye, bromophenol blue, reached the bottom of the gel. The electrode buffer was Tris-glycine, pH 8.3. After the electrophoresis the protein bands were stained with 1% Amido Schwarz in 7% acetic acid. The destaining of the gel was either performed electrophoretically or by diffusion in 7% acetic acid for about 24 hr.

The molecular weight of native nitrite reductase using gel electrophoresis was determined according to the method described by Hedrick and Smith (276). Separation gels with various concentrations of acrylamide (6-12%) were prepared according to Ornstein and Davis (277) except that the ratio of acrylamide to bis (N,N'-methylenebisacrylamide) was 30:1 which was maintained constant in all the gels. The use of spacer gel was found unnecessary. Samples (100 μl) in 5 mM Tris-glycine buffer, pH 8.2 containing 50% glycerol and 0.05% bromophenol blue were layered on top of the gels. Electro-

phoresis was carried out at 2 mA for 30 min and 4 mA for 2 hr. in a cold room (4°C). At the end of the run the dye front was marked by inserting 25 gauge copper wire. The staining and destaining were performed as already described. Migration of dye and protein bands was measured on a illuminated box using a magnifying glass mounted on the top of the light box. Measurements were accurate to ± 0.5 mm.

SDS-gel electrophoresis containing 0.1% SDS was carried out as described by Shapiro et al.(278) except that samples after treatment with 1% SDS and 1% 2-ME at pH 7.0 were incubated at 37°C for about 4 hr and were not dialyzed prior to electrophoresis. The electrophoresis was carried out at 8 mA per tube for 3 hr.

N-terminal analyses:

N-terminal amino acid residue was determined as dansyl derivative using the technique described by Gros and Labouesse (279). Protein (about 1 mg) was dansylated in 60 mM phosphate buffer pH 8.5 containing 4 M urea for 30 min at 30°C. The dansylated protein was precipitated with 10% TCA; the precipitate was recovered by centrifugation and washed twice with 1 M HCl. The dansyl-enzyme was hydrolyzed with 5.7 M HCl at 110°C for 4 hr; the hydrolyzate was taken to dryness, and the residue was suspended in 0.1 ml of a mixture of acetone-1N HCl (9:1, v/v). Thin layer chromatography on silica gel plates was used for identification of dansylamino acids using solvent system A of Morse and Horecker (280) and the solvent system b of Deyl and Rosmus (281). Migrations were compared with

standard dansylamino acids (Sigma) as necessary. N-terminal residues of glutathione and lysozyme were determined as a check on the accuracy of the method and were found to be the same as reported in the literature. The quantitative determination of N-terminal amino acid residue was performed according to the procedure of Gros and Labouesse (279) with slight changes. The dansylation of the enzyme (10 to 12 nmoles) and its hydrolysis was carried out as already described for qualitative experiments. Chromatography was performed in solvent system A of Morse and Horecker. The fluorescent spot was drawn up from the plate and the dansylmethionine was eluted three times with 0.5 ml of chloroform-methanol-acetic acid (7:2:2) mixture. The eluates were combined, evaporated to dryness and dissolved in 5 ml of absolute ethanol. The flaurescence of the samples was measured in a Beckman DU Spectrophotometer equipped with a flouresceence attachment provided with a 365 nm entrance filter. A standard dansylmethionine solution was used as a reference. Control experiments with methionine gave dansylmethionine in about 50-60% of the theoretical yield.

Amino acid analyses:

The amino acid analyses of nitrite reductase were performed without prior removal of heme groups. To prepare acid hydrolysates, lyophilized samples of the enzyme (approximately 1-2 mg protein) were heated with three-times glass-distilled constant boiling HCl at 110°C for 18,24,30 and 48 hr in evacuated and sealed Pyrex tubes in a block heater as described by Moore and Stein (282). The hydrolysates were evaporated to dryness

in a rotary flash evaporator at 45°C. The residual HCl in the hydrolysates was removed by dissolving the residue in about 2 ml of deionized water and taken to dryness by flash evaporation. This was repeated two times. The residues were taken up in citrate buffer, pH 3.8, and aliquots were analyzed in a Spinco model 120-B automatic amino acid analyzer by the method of Spackman et al. (283).

The amide content of the enzyme was determined from the amount of ammonia liberated during acid hydrolysis of the enzyme. No separate determination were performed because of inssufficient amount of the enzyme available.

Determination of sulfhydryl groups:

The free thiol groups of nitrite reductase were determined by titration of the enzyme in the presence and absence of denaturing agents (8M urea, and 8M urea plus 1% SDS) with p-HMB and DTNB. When denaturing agents were used, the protein was initially incubated with these solutions for 60 min prior to the addition of DTNB or p-HMB.

a) p-HMB titration

Titrations of the enzyme with p-HMB were carried out essentially as described by Benesch and Benesch (284). 8-9 mg of p-HMB (sodium salt) is dissolved in 1 ml of 0.04 M NaOH and solution made to 25 ml. p-HMB solutions were standardized both spectrophotometrically by direct optical density determination at 232 nm (ε_M = 1.69 x 10^4) (250) and by titration against standard reduced glutathione solution as described by

Benesch and Benesch (284). The determinations by the two methods were in good agreement.

Titrations of nitrite reductase samples were carried out on accurately measured aliquots of the protein in 1 ml stoppered silica cuvettes of 10 mm light path. The protein samples were taken in 50 mM potassium phosphate buffer, pH 7.0. Small aliquots of standard p-HMB solution were added to the experimental solution and the blank which contained equal volume of buffer. The contents were mixed and the optical density measured at 255 nm after each addition till there was no further change. The observed optical densities were corrected for dilution and plotted against the volume of the p-HMB added. The end point is obtained from the intersection of the two lines as shown in Fig. 15

b) DTNB titration:

The titrations of the enzyme with DTNB were carried out according to the procedure described by Thorner and Paulus (285). Appropriate dilutions of the enzyme were prepared in 1 ml volume in 1-ml capacity silica cuvettes of 10 mm light path and the titration was started by the addition of 0.02 ml of 10 mM DTNB in 0.05 M potassium phosphate buffer, pH 7.5. The reaction was monitored at 412 nm with a Beckman DU Spectrophotometer over a period of about 6-8 hr. The same procedure was used when the titrations were performed in the presence of the denaturing agents except that the reaction was complete within about an hour's time. An extinction coefficient of 13,600 (286) was used for reduced thionitrobenzoate for calculating the

free thiol groups. The accuracy of the method was checked with reduced glutathione.

Determination of total sulfhydryls and disulfide bonds :

Total -SH groups and -S-S- linkages in nitrite reductase were determined by two different procedures:

1) Reduction of -S-S-linkages with $NaBH_4$ in 8M urea followed by DTNB titrations after removal of excess of $NaBH_4$.

2) Oxidation of -SH and -S-S- groups to cysteic acid by the standard procedure of performic acid oxidation followed by acid hydrolysis and cysteic acid estimation using an amino acid analyzer.

1) Reduction of by $NaBH_4$ followed by DTNB titration:

The reduction and estimation were carried out by a slight modification of the procedure of Cavallini, Graziani and Dupre (287). Test tubes (18 x 110 nm) with marks at 3 ml and 6 ml were used for this experiment. The following were added to the tubes in the order shown: 1.44 g of solid urea, 0.1 ml of 0.1 M di Na-EDTA, 1 mg of the purified enzyme, 1 ml of 2.5% $NaBH_4$ prepared just before use and water to make upto 3 ml. The tubes were shaken in order to dissolve urea and incubated at 38°C for 45 min. 0.5 ml of 1 M KH_2PO_4 containing 0.2 N HCl was then added. The destruction of $NaBH_4$ was completed by adding 2 ml of acetone. The solution was shaken thoroughly and brought well into contact with the walls of the test tube. Nitrogen was passed through the solution and 0.1 ml of 0.1 M DTNB was added under nitrogen. After standing for 15 min the light

absorption at 412 nm was determined. The number of sulfhydryl groups (N) was calculated using the following formula.

$$N = \frac{Mw \times A \times V}{12000 \times m}$$

where Mw = molecular weight of the protein

A = absorbancy

V = volume of the final solution

m = weight in mg of the protein sample analyzed

The accuracy of the method was checked with bovine serum albumin and lysozyme as standard.

2) Performic acid oxidation and cysteic acid estimation:

The total half-cystine content was determined as cysteic acid after oxidation with performic acid according to Moore (288) followed by hydrolysis and amino acid analysis as above. The performic acid treatment removes heme moieties of the heme protein (289). This method will, therefore, also estimate the cysteine residues which are bound to heme moieties of the protein.

Estimation of tyrosine and tryptophan:

Tyrosine and tryptophan were determined both spectrophotometrically by the method of Benesz and Schmid (290) and Goodwin and Morton (291) and colorimetrically by the method described by Uehara et al. (292) for tyrosine and that of Spies and Chambers (293) for tryptophan. The values of tryptophan and tyrosine obtained by these methods were in good agreement.

Goodwin and Morton's method:

Proteins show selective absorption in the ultraviolet region and the position of the absorption maximum varies with pH. The majority of the constituent amino acids do not show any absorption in the region 250-320 nm and it is known that phenylalanine, tyrosine and tryptophan are responsible for the observed ultravielet absorption of protein solutions. In 0.1 N NaOH the absorption by tyrosine and tryptophan is much stronger and that by phenylalanine is negligible. Under these conditions the protein solutions may be treated as a two-component system for spectrophotometric analysis. The intensity of absorption at the point where the curves for tyrosine and tryptophan intersect is a direct measure of the total molar solute concentration and will be the same however the proportions are varied. At other wave lengths the intensity of absorption will vary with the relative proportions of the components. Using 0.1 N NaOH as solvent the two absorption curves intersect at 294.4 nm (ε = 2375) and 257.15 nm (ε = 2748). By determining the absorption of the protein in 0.1 N NaOH at the above two wavelengths and at one other wavelength (e.g. at 280 nm) it is possible to determine the relative proportions of tyrosine and tryptophan in the protein.

Thus, if x = total mole/l in solution

y = g mole of tyrosine

x-y = g mole of tryptophan

At any wavelength other than the point of intersection let

ε tyrosine be A, and ε tryptophan be B and the observed intensity of absorption for a 10 mm cell E

then,

$$E = yA + (x-y)\,B$$

$$\text{or} \quad Y = \frac{E - xB}{A - B}$$

$$x = \frac{\text{E value at an intersection}}{\varepsilon\text{tyr at an intersection}}$$

(2) Benoze and Schmid's method (graphical method):

This method is based upon measuring the absorbance of the protein in 0.1 N NaOH in the range between 278 and 294 nm at 2 nm intervals. The readings are plotted against the wavelength and a line is drawn tangentially to the two characteristic peaks. From the slope of the tangent, the maximum absorption between 270 and 290 nm, and the molecular weight of the protein the tyrosine and tryptophan content is determined.

Attempts to use heme-free enzyme in the determination of tyrosine and tryptophan by spectrophotometric methods were not successful as heme-free enzyme /heme split by Paul's procedure (294)/ tend to precipitate in alkaline solutions. The heme of nitrite reductase absorbs more or less uniformly in the range 272 to 292 nm and is therefore not likely to interfere with the characteristics of the slope of the tangent used in Benoze and Schmidt's method to compute the ratio between tyrosine and tryptophan. A correction for absorption due to heme was however applied to the value of absorption maximum which estimates the total x tyrosine and tryptophan

content. A similar correction was applied in Goodwin and Morton's method.

(3) Colorimetric determination of tyrosine:

Tyrosine content of nitrite reductase was also determined by the colorimetric method of Uehara, Mannen and Kishida (292). The method involves alkali-denaturation of protein in a boiling water bath, the color forming reaction between tyrosine and 1-nitroso-2-naphthol in 19 N H_2SO_4, and measurement of the absorbance at 520 nm. The procedure is as follows:

Nitrite reductase (0.6 - 1.5 mg) in water (1 ml) was added to 1 ml of 0.15% (w/v) 1-nitroso-2-naphthol in 0.1 N NaOH and 2 ml of a mixture of equal volumes of 0.025 N HNO_3 and 0.3 N NaOH. The mixture was heated in a boiling water bath for 10 min and then placed in a water bath at 50°C until equilibrium was reached. Concentrated H_2SO_4 (4 ml) was then added. The red color was measured after 15 min against a reagent blank at 520 nm. Tyrosine standards were run at the same time. The accuracy of the method was checked with ovalbumin and ribonuclease.

(4) Colorimetric determination of tryptophan:

Tryptophan was determined by colorimetric method (procedure K) of Spies and Chambers (293). Eight milliliters of 23.7 N H_2SO_4 and 1 ml of 2 N H_2SO_4 containing 30 mg of p-dimethylaminobenzaldehyde were mixed and cooled to 25°C. To this solution is added 1 ml of aqueous enzyme sample. The solution was mixed, cooled to 25°C, and kept for 12 hr. To the solution was then added 0.1 ml of an 0.045% solution of

$NaNO_2$. After 30 min, absorbance is read and converted to weight of tryptophan from the standard curve prepared in a similar way. The accuracy of this method was checked with bovine serum albumin and ovalbumin.

Determination of degree of hydrophobicity:

The degree of hydrophobicity of A. fischeri nitrite reductase and nitrite reductases from P. aeruginosa and Chlorella fusca was calculated from their amino acid compositions. Three different methods were followed.

(1) Fisher's method:

According to Fisher (295) the degree of hydrophobicity is expressed in terms of a polarity ratio, p, which is defined by the following equation

$$p = V_e / V_i$$

where V_e and V_i are the volumes occupied by polar and nonpolar residues, respectively. Arginine, histidine, lysine, aspartic acid, glutamic acid (and their amides), tyrosine, serine and threonine were considered by Fisher (295) as polar residues and all other amino acids as nonpolar residues.

(2) Waugh's method:

According to the method of Waugh (296) the hydrophobicity is measured in terms of NPS, the frequency of nonpolar side chains. NPS is calculated by counting the tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine and valine residues and expressing the sum as a fraction of the total number of residues.

(3) Bigelow's method:

Bigelow's method (297) gives average hydrophobicity, $H\phi_{av}$, which is based on Tanford's (298) free energies of transfer of amino acid side chains from an organic environment to an aqueous environment. $H\phi_{av}$ is the total hydrophobicity divided by the total number of residues.

Determination of isoionic point:

The isoionic point of A. fischeri nitrite reductase was determined from the titration curve which was constructed on the basis of amino acid composition. Calculations for constructing the theoretical titration cruve were carried out according to Cohn and Edsall (299) and were based on the assumption that there are no electrostatic interactions between ionizable groups and that each member of each species is identical, ionizing independently. The principle of calculation for theoretical titration curve is as fallows.

If n_1 is the number of ionizable acid groups of class I and α_1 the fraction of group in the ionized state at a particular pH, the number of negatively charged groups (class I) is given by the following expression.

$$n_1 \cdot \alpha_1$$

α_1 as a function of pH can be evaluated by the equation

$$pH = pK + \log \frac{\alpha_1}{1 - \alpha_1}$$

where pK is the negative log of ionization constant of the respective ionizable group. In the case of basic groups,

however, the number of positively charged groups is given by

$$n_2 (1 - \alpha_2)$$

where n_2 is the number of basic groups (Class II) and α_2 is the fraction of the groups that has ionized at a given pH.

Since proteins are polyvalent ampholytes containing a large number of acid and basic groups of more than one type, the total mean net charge (Z) at a particular pH is given by the difference between the sum of negatively charged and the sum of positively charged groups.

$$Z = \left[n_{Arg} (1-\alpha_{Arg}) + n_{Lys} (1-\alpha_{Lys}) + n_{His} (1- \alpha_{His})\right] -$$

$$\left[n_{carboxyl} \times \alpha_{Carboxyl} + n_{Tyr} \times \alpha_{Tyr} + n_{Cys} \times \alpha_{Cys}\right]$$

α and n have the same meaning as described above.

The net charge on the enzyme protein between pH 2 and pH 14 was calculated by the use of the above equations and the values were plotted against pH, resulting in a theoretical titration curve. The pK values for different acid and basic groups were taken from Mahowald, Neltmann, and Kuby (300). ~~Neltmann (300).~~

Chapter 3

PURIFICATION

SUMMARY

Nitrite reductase has been purified from Achromobacter fischeri by a modification of an earlier procedure of Prakash and Sadana (170). The enzyme has been obtained, for the first time, in homogeneous form as judged by ultracentrifugation and polyacrylamide disc gel electrophoresis. The overall recovery of the enzyme was 31% as compared with 15-17% obtained by the earlier procedure. The purification procedure consisted of extraction, isoelectric precipitation at pH 4.5, protamine sulfate treatment, fractionation with ammonium sulfate, two successive hydroxylapatite chromatography steps, and a simplified preparative gel electrophoresis.

The purified enzyme has a specific activity of about 150-155 μmoles NO_2^- reduced per min per mg protein with benzyl viologen as electron donor. Methyl viologen could also serve as an electron donor and was twice as effective as benzyl viologen.

INTRODUCTION

The purification of bacterial nitrite reductases has been attempted from a number of sources. Nitrite reductases thus far prepared from P. stutzeri (143), P. aeruginosa (145,162), P. denitrificans (163), E. coli Bm (161), E. coli K12 (114), M. denitrificans (110), N. europaea (151), A. faecalis (112) and A. cycloclastes (150) were of various degrees of purity. Although the enzymes reported from P. aeruginosa (162),

P. denitrificans (163), M. denitrificans (110), A. faecalis (112) and A. cycloclastes (150) were highly purified none has been obtained in a form which is homogeneous both in the ultracentrifuge and in the disc gel electrophoresis. The enzyme preparation reported by Horio et al. (111) from P. aeruginosa was approximately 70% pure as judged by diffusion and sedimentation patterns. Yamanaka et al. (162) modified the procedure of Horio et al.(111) and obtained a crystalline preparation of the enzyme. The homogeneity of this preparation was not determined by any other criteria. The enzyme preparation purified from P. denitrificans by Iwasaki et al. (163) was homogeneous in the ultracentrifuge. The purification of M. denitrificans nitrite reductase was reported by Newton (110). The enzyme preparation showed slight impurity as judged by its behaviour on cellulose acetate and polyacrylamide gel electrophoresis. More recently, Iwasaki and Matsubara purified nitrite reductases from A. faecalis (112) and A. cycloclastes (150). The purified enzyme from both these sources showed slight amount of impurity as revealed by disc gel electrophoresis.

The purification of nitrite reductase from A. fischeri was first reported from this laboratory by Prakash et al. (49). The purification procedure consisted of preparation of crude extract, isoelectric precipitation, protamine sulfate treatment, ammonium sulfate fractionation and chromatography on hydroxylapatite gel and DEAE-cellulose. The purified enzyme was homogeneous in the ultracentrifuge. However, when checked for homogeneity by polyacrylamide disc gel electrophoresis, two

additional bands were noticed by me (301).

The work presented in this chapter describes a modified procedure for the purification of A. fischeri nitrite reductase. The last step in the purification procedure of Prakash and Sadana (170)-column chromatography on DEAE-cellulose- has been deleted, as considerable enzyme losses (40% to 70%) occur at this step. Further purification of the enzyme was carried out by a simplified preparative polyacrylamide gel electrophoresis. This has resulted in obtaining an enzyme which is homogeneous both in the ultracentrifuge as well as in the disc gel electrophoresis with an overall yield of about 31% as compared with 15-17% obtained by the procedure of Prakash and Sadana (170). The specific activity of the enzyme increased 1.4 fold to a final value of about 1500-1550 units (μmoles nitrite reduced per 10 min) per mg protein, the highest reported so far for any nitrite reductase.

The results show that reduced methyl viologen could also serve as an electron donor for the reduction of nitrite by A. fischeri nitrite reductase and that it was nearly twice as effective as benzyl viologen.

The results presented in this chapter have been published (Mazhar Husain and J.C. Sadana (1972) Analytical Biochemistry 45, 316).

Purification of A. fischeri nitrite reductase

Unless otherwise mentioned, all steps were carried out at 0-4°C.

Step 1: Preparation of crude extract:

The frozen cells (200 g) were thawed overnight at 4°C and lysed in cold distilled water (1 g wet wt/20 ml water). The suspension was stirred for 30 min, homogenized in a Potter-Elvehjem glass homogenizer, and stirred again for 30 min. The cell-free supernatant fluid was collected by centrifugation for 20 min at 44,000 x g in a Model L Spinco preparative ultracentrifuge. The supernatants were combined and the total volume of this crude extract was 3750 ml.

Step 2: pH 4.5 sediment:

The pH of the clear supernatant was adjusted to 4.5 by adding an equal volume of 0.2 M acetate buffer, pH 4.3, with constant gentle stirring. The resulting precipitate was collected by centrifugation at 4,000 x g for 30 min, resuspended in about one-third of the original volume of 50 mM potassium phosphate buffer and dialyzed against the same buffer overnight. Although, practically no purification is achieved in this step, it helps in reducing the volume of crude extract for easy handling in further purification steps.

Step 3: Protamine sulfate treatment:

The isoelectric precipitate contained large amount of nucleic acids as indicated by the optical density ratio at 280 to 260 nm and approximately 8 mg protein per ml. The enzyme was precipitated from the dialyzed clear supernatant by the addition of protamine sulfate (15 mg/ml, pH 5.0). The addition

of protamine sulfate was continued until no further precipitate was formed; about 500 ml of protamine sulfate was required. The precipitate was collected by centrifugation at 4,000 x g. The clear supernatant which had little (0.5 - 1.0%) or no nitrite reductase activity contained most of the NAD(P)H-flavin reductase.

Step 4: Extraction of nitrite reductase from protamine sulfate:

The precipitate from step 3 was suspended in 100 ml of 0.2 M potassium phosphate buffer (pH 7.5), homogenized in Potter-Elvehjem glass homogeniser, stirred for 45 min and centrifuged at 14,000 x g. The extraction was repeated 5 to 6 times in a similar manner until the extract showed negligible enzyme activity. The extracts were combined, centrifuged and the inactive precipitate was discarded. The ratio of light absorption at 280 nm to that at 260 nm increased from 0.68 in the crude fraction to 1.1. A 4-fold purification was achieved in this step.

Step 5: Ammonium sulfate fractionation:

The combined protamine sulfate extracts (590 ml) from step 4 were brought to 0.55 saturation by the addition of 192 gm of ammonium sulfate. No pH adjustment was made during ammonium sulfate fractionation. The suspension was stirred for 30 min at 4°C and centrifuged at 20,000 x g for 15 min in a Sorvall centrifuge. The sediment was discarded. The concentration of the supernatant fluid was then raised to 0.85 saturation by adding 20.7 gm of solid ammonium sulfate for every 100 ml of solution. The suspension was stirred and centrifuged as before.

The precipitate was dissolved in 50 ml of 0.02 potassium phosphate buffer (pH 6.8) and dialyzed overnight against the same buffer with three changes of the buffer.

Step 6: First chromatography on hydroxylapatite column:

The dialyzed enzyme solution (1317 mg) was passed through a hydroxylapatite column (1.6 x 50 cm) which was previously equilibrated with 0.02 M potassium phosphate buffer (pH 6.8). The column was washed with the same buffer until the absorbancy of the washings was less than 0.01 at 280 nm. Nitrite reductase was adsorbed on the column as a pink diffused band and remained stationary while the column was being washed with 0.02 M potassium phosphate buffer (pH 6.8). About 400 ml of this buffer was used. The column was then washed with 0.05 M potassium phosphate buffer (pH 6.8) and the washing was continued until nitrite reductase activity started appearing in the eluate. About 400 ml of the buffer was required. The enzyme was eluted with 0.2 M potassium phosphate buffer (pH 6.8) at a flow rate of 15-20 ml/hr and fractions of 2-5 ml volume were collected. The highly active enzyme fractions were combined and dialyzed overnight against 0.02 M potassium phosphate buffer (pH 6.8) with three changes of the buffer.

Step 7: Second chromatography on hydroxylapatite column:

The combined dialyzed fractions containing 300 to 400 mg protein were applied to a second hydroxylapatite column (1 x 40 cm), equilibrated, developed and eluted as before.

The pink nitrite reductase band was eluted as one major fraction (5 ml) which contained 155 mg of protein. The enzyme was dialyzed overnight against 300 ml of 0.05 M potassium phosphate buffer (pH 6.8) with three changes of the same buffer. The dialyzed enzyme was either used immediately for further purification or stored at -15°C until used. The specific acitivity of the enzyme at this stage was about 680-700 units (≠ μmoles NO_2^- reduced per 10 min) per mg protein and the recovery was approximately 50% of the initial activity present in the crude extract.

Step 8: Preparative polyacrylamide gel electrophoresis:

When the purified enzyme from the preceding step was subjected to disc gel electrophoresis, the preparation gave one major and four minor protein bands (Fig. 2a). The major band which contained about 40-50% of the total protein applied was coincident with nitrite reductase. Three of the impurity bands were slow moving and one fast moving when compared to the enzyme band.

Further purification of the enzyme by chromatography on DEAE-cellulose resulted in about 60-70% loss of the enzyme activity (170). Also the enzyme after DEAE-cellulose chromatography was found polydisperse in the disc gel electrophoresis retaining one slow and the fast moving impurity (Fig. 2b). In order to avoid the heavy losses of enzyme activity during DEAE-cellulose chromatography, attempts were made to use Sephadex G-100 and DEAE-Sephadex A-50 instead of DEAE-cellulose. No encouraging results were obtained.

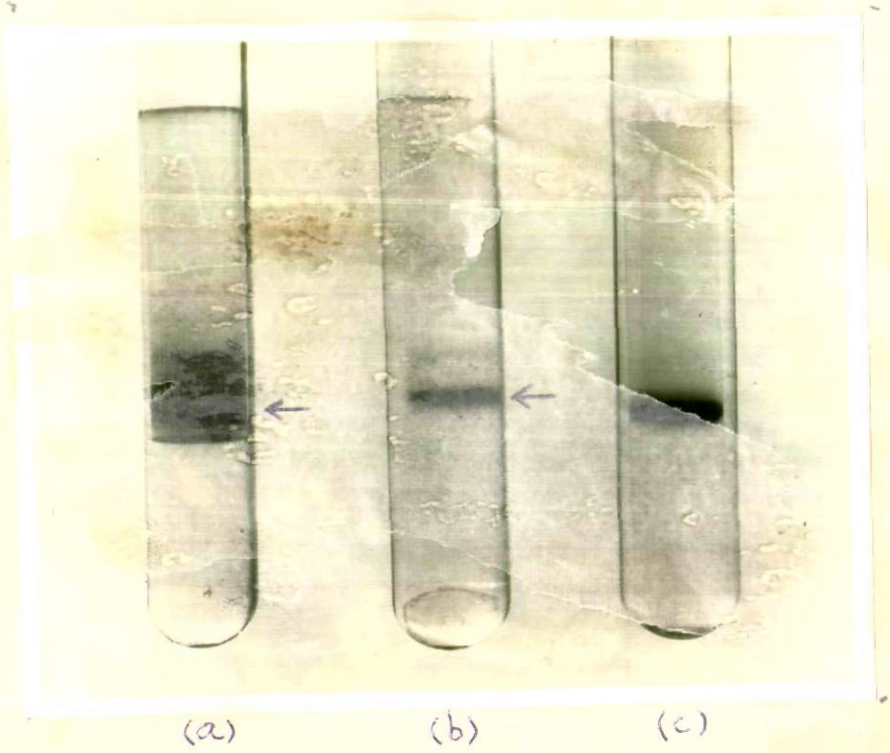


FIG. 2 DISC GEL ELECTROPHORETIC PATTERN OF NITRITE REDUCTASE FROM VARIOUS PURIFICATION STAGES.

7.5% gels with tris-glycine system, pH 8.6, were used. (a) Enzyme fraction obtained after second hydroxylapatite column chromatography. (b) Fraction a purified by DEAE-cellulose chromatography. (c) Fraction a purified further by preparative gel electrophoresis. Arrows indicate the location of the enzyme. Migration took place from the top (-) to bottom (+). Other conditions were as described under Materials and Methods.

Purification of the enzyme to homogeneity was, however, achieved by the use of preparative polyacrylamide gel electrophoresis.

A simplified procedure of preparative polyacrylamide gel electrophoresis was adopted for further purification of the enzyme. The preparative electrophoresis was performed in 7.5% acrylamide gel at pH 8.3 - 8.5. The apparatus used for carrying out the preparative electrophoresis resembled that described by Davis (275) consisting of electrode vessels provided with platinum electrodes. The cathode vessel was also provided with two ground glass ǂ joints (19 B) for attaching the gel columns. The electrophoretic columns (2 x 15 cm) were filled with acrylamide gel solution prepared according to the procedure described by Davis (275) and Ornstein (277) to the top, leaving about 2-4 ml volume for applying the enzyme samples. The gels were polymerized using 0.04% ammonium persulfate. After polymerization was over, residual persulfate was removed by passing a current of about 10 mA through the column for 4 hr with cathode at the top of the column. Tris-glycine buffer was used both during the washing of the gel columns as well as during the preparative electrophoresis.

The purified enzyme obtained after second hydroxylapatite column chromatography (Step 7) was dialyzed against 0.01 M potassium phosphate buffer (pH 7.5) for about 6-10 hr with three changes of the buffer. Enzyme samples (1-2 ml) containing 20-30 mg protein were made 20% in sucrose and layered on the top of each column. Electrophoresis was

commenced and was carried out at 4°C at a constant current of about 6 mA per column. In order to avoid excessive heating, the columns were kept immersed in lower bath buffer. The enzyme under these conditions migrates as a sharp band and being red in color was easily detectable visually without staining the gel. As the run progressed a faint pink band which represents one of the impurities, was seen separating from the enzyme band. After about 5-6 hr, the apparatus was switched off and a piece of dialysis sacking filled with 1.5 ml of bath buffer (tris-glycine, pH 8.3) was attached to the lower end of the column. Trapped air bubbles, if any, were removed by introducing a plastic capillary into the dialysis sacking by the side of the column and pushing the sacking upward. The electrophoresis was continued till the red enzyme band reached the end of the column. The main enzyme band was eluted into a fresh dialysis sacking containing tris-glycine buffer. Care was taken to avoid mixing of other proteins with the major enzyme fraction. The remaining slow moving proteins were collected as a third fraction. At the end of electrophoresis, the three fractions were dialyzed overnight against 300 ml of 0.05 M potassium phosphate buffer (pH 6.8) with three changes of the buffer and the activity and protein determined. Three preparative runs using two columns at a time were carried out to purify all the enzyme obtained from second hydroxylapatite column (Step 7).

The results of preparative polyacrylamide gel electrophoresis are presented in Table 3. About 85-90% of the total

TABLE 3

PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHOREIS OF *A. FISCHERI* NITRITE REDUCTASE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units x 10^{-3}	mg/ml	mg	units/mg protein	%
Second hydroxyl-apatite eluates	5.0	21,200	106	31	155	684	100
Eluted fractions (combined)							
1	4.0	2125	8.5	7.5	30	283	8.0
2	4.8	14,580	70.0	9.6	46	1520	66.0
3	4.5	3178	14.3	13.8	62	230	13.5
Total combined fractions	13.3		92.8		138		87.5

enzyme activity was recovered in all the three fractions with most of the activity (66%) in the major enzyme fraction. On protein basis a recovery of about 90% is recorded. The final specific activity of the enzyme (main enzyme fraction) increased from 700 to 1520 units/mg protein indicating a purification of about 2.2-fold in this final step. The electrophoretic behaviour of the enzyme obtained after second hydroxylapatite, DEAE-cellulose and preparative polyacrylamide gel columns is shown in Fig 2.

In the preliminary purification studies, the colored enzyme band was cut at the end of electrophoresis and eluted by homogenizing and centrifuging. This was, however, found inconvenient and resulted in low recoveries (40-50%).

The purification of the enzyme by preparative gel electrophoresis has been repeated 8-10 times with reproducible results. A summary of the purification procedure is given in Table 4. The yield of the final purified enzyme is about 46 mg protein from 200 g of bacterial cells (wet weight) and on a protein basis represents a 84-fold purification, with an overall recovery of 31%. The overall recovery of the enzyme of 31% by the present procedure represents a 2-2.5-fold increase over that of 12-15% by the procedure of Prakash and Sadana (170). The specific activity of the enzyme also increased to about 1.5 fold to a value of 1520 units per mg protein.

Homogeneity:

The homogeneity of the purified enzyme of highest specific activity was examined both by polyacrylamide disc gel electro-

TABLE I

PURIFICATION PROCEDURE OF *A. FISCHERI* NITRITE REDUCTASE FROM 200 g (WET WEIGHT) OF BACTERIA

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units x 10^{-3}	mg/ml	mg	units/mg protein	%
Crude extract	3750	60	225	3.3	12375	18.2	100
pH 4.5 sediment	1280	158	202	8.4	10752	18.8	90
Phosphate extract of protamine sulfate-treated sediment	590	305	180	4.1	2419	74	80
0.55 - 0.85 satd. $(NH_4)_2SO_4$ ppt.	85	1810	154	15.5	1317	117	68
First hydroxylapatite eluates	19	6895	131	18.0	342	383	58
Second hydroxylapatite eluates	5	21200	106	31.0	155	684	47
Polyacrylamide gel electrophoresis	4.8	14580	70	9.6	46	1520	31

phoresis and ultracentrifugation. The enzyme sedimented as a single symmetrical peak at 59,780 rpm in the ultracentrifuge. The patterns obtained after 18,32,40,48 and 56 min of ultracentrifugation indicated that the purified enzyme was homogeneous (Fig. 4, see chapter IV).

The examination of homogeneity by polyacrylamide disc gel electrophoresis was carried out using 7.5% gels. About 30-50 μg of protein was used. The enzyme migrated towards the anode as a sharp single red band which coincided with the protein band visualized on staining (Fig. 3).

Apparent and real specific activities of purified nitrite reductase

Protein determinations on purified enzyme preparations were routinely carried out by the method of Lowry et al. (257). However, when the estimations were made by optical (258,259) and micro-Kjeldahl (262) methods (ref. chapter II), it was observed that the values obtained are lower than that determined by Lowry's method. It was, therefore, thought necessary to evaluate the accuracy of the three methods of protein determination. Protein estimations on bovine serum albumin, mammalian cytochrome c and purified nitrite reductase were performed by the different methods; the results are compared in Table 5. Although the three methods gave different results, the values obtained for bovine serum albumin and cytochrome c by the micro-Kjeldahl method were in close agreement with those determined from absorption coefficients at their specific wavelengths (ref. chapter II). The protein

FIG. 3 DISC GEL ELECTROPHORESIS OF PURIFIED NITRITE REDUCTASE.

About 50 ug enzyme protein was used. Electrophoresis was carried out in 7.5% gel with tris-glycine system, pH 8.6 at 3 mA/gel. Migration was towards the anode (bottom). Other conditions were as described in Materials and Methods.

TABLE 5

DETERMINATION OF PROTEIN BY DIFFERENT METHODS ON SAMPLES OF BOVINE SERUM ALBUMIN MAMMALIAN CYTOCHROME c AND PURIFIED ACHROMOBACTER FISCHERI NITRITE REDUCTASE

Protein	Protein per ml (mg)				
	By weight	From specific absorption*	Optical method (258,259)	Method of Lowry et al. (257)	Micro-Kjeldahl method (262)
Bovine serum albumin	1.0	1.0	0.61	-	0.94
Cytochrome c	1.0	0.94	0.38	1.28	0.95
Nitrite reductase	-	-	7.34	9.6	8.64

*The concentrations of bovine serum albumin and cytochrome c were determined from their extinction coefficients at 280 nm (260) and 550 nm (261) respectively.

values obtained by the optical method were lower and by the Lowry's method higher as compared to those obtained by the micro-Kjeldahl method. Finally, the comparison of areas of sedimenting enzyme protein in synthetic boundary cell and that of the standard albumin solution under similar conditions gave a value which was in agreement with the value obtained by the micro-Kjeldahl method indicating that the latter method is more accurate than that of Lowry's.

Based on the protein values determined by micro-Kjeldahl method, the specific activity of purified (electrophoretically homogeneous) nitrite reductase was calculated to be 1688 units per mg protein in contrast to a value of 1520 units/mg protein by the method of Lowry et al. The former, therefore, is the true specific activity of the enzyme.

Methyl viologen as electron donor:

Table 6 shows that nitrite reductase from A. fischeri could also use MVH as electron donor. In experiments with MVH as electron donor, the assay procedure for nitrite reductase was the same as described for BVH. MVH was found to be a more effective electron donor than BVH. The specific activity of the enzyme with MVH was about two times more than that obtained when BVH was used as electron donor. Similar observations have been reported in the case of nitrite reductase from yeast, T. nitratophila (70), algae (125, 134), spinach (164), and cultured tobacco cells (302).

Unless otherwise stated, the enzyme concentrations and specific activities mentioned in the present work will refer

TABLE 6

USE OF METHYL VIOLOGEN AS ELECTRON DONOR FOR A.FISCHERI NITRITE REDUCTASE

The incubation mixture contained in a final volume of 3.0 ml: potassium phosphate (pH 7.5), 200 μmoles; $NaNO_2$, 0.6μmole; enzyme, 0.2 μg; benzyl viologen or methyl viologen, (10 mg/ml), 0.5 ml; sodium dithionite, 1 mg in 1 ml of 0.2 M potassium phosphate (pH 7.5). Viologen dyes and sodium dithionite were taken in the side arm of the Thunberg tubes. Reaction was carried out for 5 min at 30°C. The disappearance of nitrite was followed on 1 ml samples by diazo-coupling procedure of Snell and Snell (253) as described in Chapter II.

Electron donor	Nitrite reduced/10' (μmoles)
Benzyl viologen	0.305
Methyl viologen	0.585

to the values obtained by Lowery's method with BVH as electron donor.

DISCUSSION

The enzyme nitrite reductase which catalyzes the reduction of nitrite to ammonia has been studied in a variety of organisms. Nitrite reductases from different sources have been purified to varying extents and some of these have been characterized. The enzymes from spinach and C. fusca have, however, been recently obtained in electrophoretically homogeneous form by Cardenas et al. (136) and Zumft (135) respectively. The specific activities of the two plant nitrite reductases, spinach and C. fusca, were respectively 33.85 and 51.7 μmoles nitrite reduced per min per mg protein with MVH as electron donor. The highly purified enzyme preparation from C. pepo (165) which showed faint impurity bands on electrophoresis had a specific activity of 46 μmoles of NO_2^- reduced per min per mg protein with reduced ferredoxin as electron donor.

There is as yet no report of a homogeneous nitrite reductase from bacteria. Crystalline preparations of nitrite reductases were obtained from P. aeruginosa (162) and A. faecalis (112). The A. faecalis enzyme revealed minor impurity bands on cellulose acetate or polyacrylamide gel electrophoresis. However, no mention was made whether the P. aeruginosa enzyme was pure by any other criteria (162).

Similarly, purified nitrite reductases from M. denitrificans (110) and A. cycloclastes (150) were not homogeneous when judged by disc gel electrophoresis.

The purification procedure described here has provided for the first time a preparation of nitrite reductase from A. fischeri which is homogeneous both in the ultracentrifuge and in the disc gel electrophoresis. The present procedure, which is a modification of the method of Prakash and Sadana (170), avoids use of column chromatography on DEAE-cellulose. The enzyme obtained after second hydroxylapatite column chromatography is directly purified by a simplified preparative gel electrophoresis procedure. The introduction of a preparative polyacrylamide gel electrophoresis in place of DEAE-cellulose chromatography resulted in a two-fold increase in the overall recovery of the enzyme (31%) as compared to the previous procedure. The final specific activity of 152 μmoles of NO_2^- reduced per min (1520 units) per mg protein is the highest reported so far. The results of the enzyme purification by the present procedure and the procedure of Prakash and Sadana (170) have been compared in Table 7.

Protein determinations on purified preparations of nitrite reductase, ~~mammalian cytochrome c and bovine serum albumin~~ by optical, micro-Kjeldahl and Lowry's methods gave variable results. The micro-Kjeldahl method based on nitrogen estimation, which gave lower values as compared to those by the Lowry's method, was found to be more reliable. A final specific activity of

TABLE 7

SUMMARY OF PURIFICATION OF A. FISCHERI NITRITE REDUCTASE FROM 200 g (WET WEIGHT) BY THE PRESENT PROCEDURE AND THAT OF PRAKASH AND SADANA (170)

Method	Last step of purification	Total activity	Total protein	Specific activity	Overall yield	Homogeneity
		units	mg	units/mg protein	%	
Prakash and Sadana	Column chromatography on DEAE-cellulose	35,000	29	1200	16	Homogeneous in the ultracentrifuge but polydisperse in disc gel electrophoresis
Present procedure	Preparative polyacrylamide gel electrophoresis	70,00	46	1520	31	Homogeneous both in the ultracentrifuge and disc gel electrophoresis

about 1688 units per mg protein was calculated for purified nitrite reductase on the basis of protein estimated by micro-Kjeldahl method.

A. fischeri can also use methyl viologen as electron donor for reduction of nitrite. The rate of nitrite reduction with MVH as the electron donor was about two times faster than that obtained with BVH. Similar observations have been made in the case of enzymes from T. nitratophila (70), algae (125,134), spinach (164) and cultured tobacco cells (302).

The simple preparative gel electrophoresis described here can be applied for the purification of colored proteins such as hemeproteins from relatively crude or partially purified preparations. The procedure provides an easy method for removing selectively colored protein-impurities from enzyme preparations. No special equipment other than the apparatus used for the analytical polyacrylamide disc gel electrophoresis is required. The technique has been used successfully in this laboratory for the purification of A. fischeri NAD(P)H-flavin reductase (170).

CHAPTER 4

MOLECULAR WEIGHT AND SUBUNIT STRUCTURE

SUMMARY

The *Achromobacter fischeri* nitrite reductase used in the present studies was monodisperse as judged by ultracentrifugation and disc gel electrophoresis. The native enzyme has a $s^{\circ}_{20,w}$ value of 5.[illegible] S and an average molecular weight of 80,000 as determined by the Archibald approach-to-equilibrium method, disc gel electrophoresis and also from a combination of hydrodynamic properties. The diffusion coefficient ($D_{20,w}$) and Stokes' radius determined by gel filtration are 6.05 F and 3.49 nm, respectively. From Stokes' radius and $s^{\circ}_{20,w}$ values, a frictional ratio of 1.25 could be calculated. In the absence or presence of 8M urea or 8M urea plus 1% SDS, four sulfhydryl groups reacted with DTNB, or p-HMB. Titration of the enzyme with DTNB after borohydride reduction in urea gave a value of 6 thiol groups indicating the presence of one disulfide bond in the enzyme.

Nitrite reductase does not dissociate in the presence of 6M Gu.HCl or 6M urea. The enzyme, however, splits into two physically indistinguishable subunits upon treatment with 6M Gu.HCl or 1% SDS in the presence of 1% B-ME. The subunit molecular weight of the enzyme, determined by the Archibald approach-to-equilibrium method in 6M Gu.HCl- 0.1M 2-ME and SDS-gel electrophoresis in the presence of 1% B-ME, was approximately 39,000. The subunits appear homogeneous in SDS-gel electrophoresis as well as in the ultracentrifuge with

a sedimentation coefficient of 1.4 S at a protein concentration of 7 mg/ml. Studies with dansyl chloride indicate that methionine is the only N-terminal amino acid. The data suggest that A. fischeri nitrite reductase is comprised of two subunits of equivalent size which are covalently bonded by a disulfide bridge.

INTRODUCTION

In a previous communication (170) from this laboratory, the ultracentrifugally pure nitrite reductase from A. fischeri was reported to have a molecular weight of 95,000 ± 4000 as determined by the Archibald approach-to-equilibrium method, and $s^{\circ}_{20,w}$ value of 5.2 S. Further investigations (ref. chapter III) showed that the enzyme preparation used in the previous work was polydisperse in polyacrylamide disc gel electrophoresis (301). It was, therefore, thought necessary to reinvestigate the molecular weight of the native enzyme which is homogeneous both in the ultracentrifuge as well as in the disc gel electrophoresis. The use of several different methods of molecular weight determinations, described in the present investigation, indicated that the prior estimate for the molecular weight is in error. The results of dissociation and molecular weight studies indicate that the native enzyme consists of two polypeptide chains which are covalently linked by a disulfide bond. Studies with dansyl chloride indicate that methionine is the N-terminal amino acid.

The data presented in this chapter have already been published (Husain, M and J.C. Sadana (1974) European Journal of Biochemistry 42, 283-289).

RESULTS

SEDIMENTATION BEHAVIOUR IN THE ULTRACENTRIFUGE

The sedimentation profiles obtained with the pure enzyme are presented in Fig. 4. The enzyme sediments as a single symmetrical peak in 50mM potassium phosphate buffer (pH 6.8), and is apparently homogeneous in the ultracentrifuge. Sedimentation coefficients determined at protein concentrations from 1.0 to 9.0 mg/ml fell on a straight line after appropriate corrections for the density and viscosity of water at 20°C. The sedimentation coefficient of the native protein exhibited a slight dependence on protein concentration as shown in Fig. 5. The extrapolated value of the sedimentation coefficient at infinite dilution ($s^{\circ}_{20,w}$) is 5.25 S. The dependence of the sedimentation coefficient on the enzyme concentration is best described by the equation.

$$s_{20,w} = 5.25\ (1-0.0029\ c)$$

where $s_{20,w}$ is the observed sedimentation coefficient corrected for density and viscosity of water at 20°C at a given protein concentration c (mg/ml).

MOLECULAR WEIGHT OF THE NATIVE ENZYME

Three different methods were used in the determination of molecular weight of the native enzyme.

(i) By ultracentrifugation

The molecular weight (Mw) of nitrite reductase was determined according to the procedure of Archibald (Fig.6) (265).

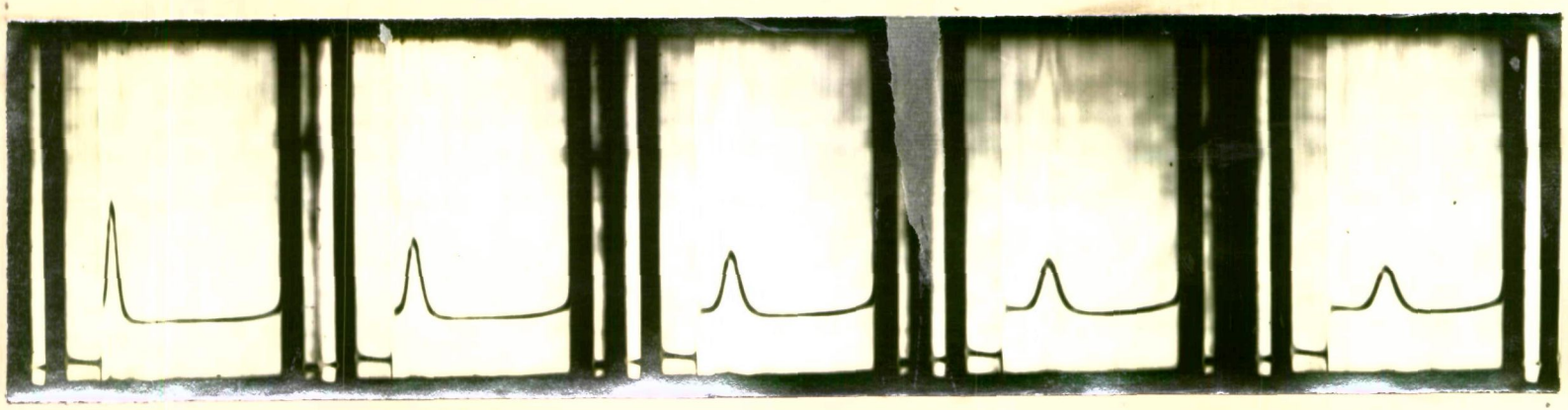


FIG. 4 ULTRACENTRIFUGE SCHLIEREN PHOTOGRAPHS OF PURIFIED NITRITE REDUCTASE

Protein concentration, 7 mg/ml; buffer, 50 mM phosphate, pH 6.8; speed, 59,780 rpm; temperature, 3.45°C; phase plate, 60°.

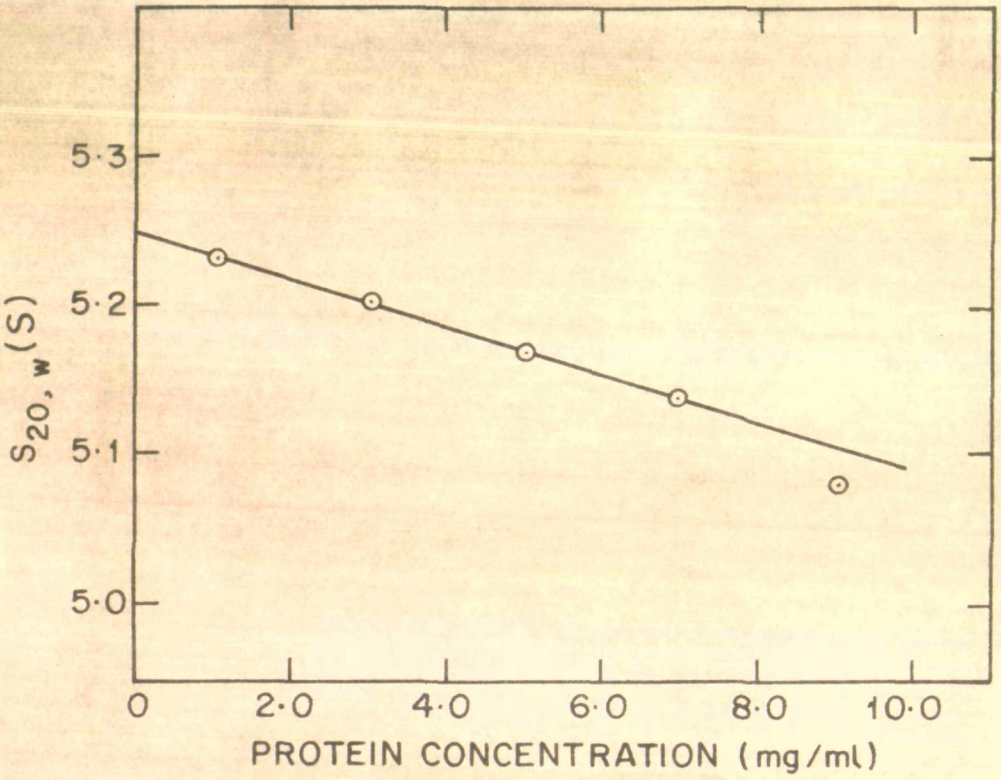
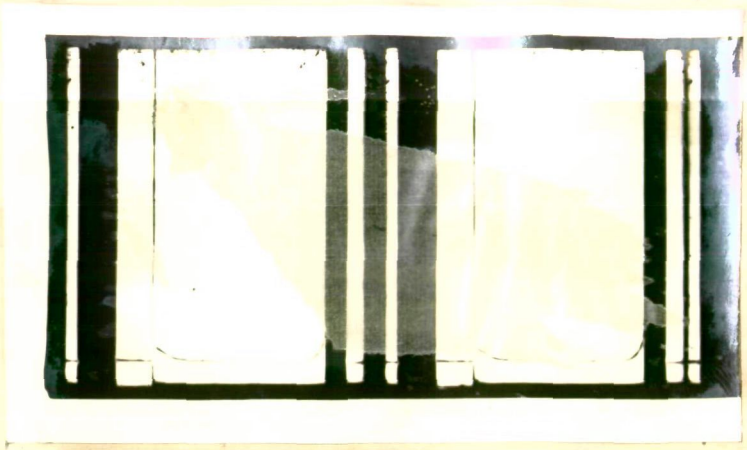


FIG. 5 **DEPENDENCE OF SEDIMENTATION COEFFICIENT OF A. FISCHERI NITRITE REDUCTASE ON PROTEIN CONCENTRATION.** The solvent was 0·05 M potassium phosphate buffer, pH 6·8.



Time(min) 60 75

a

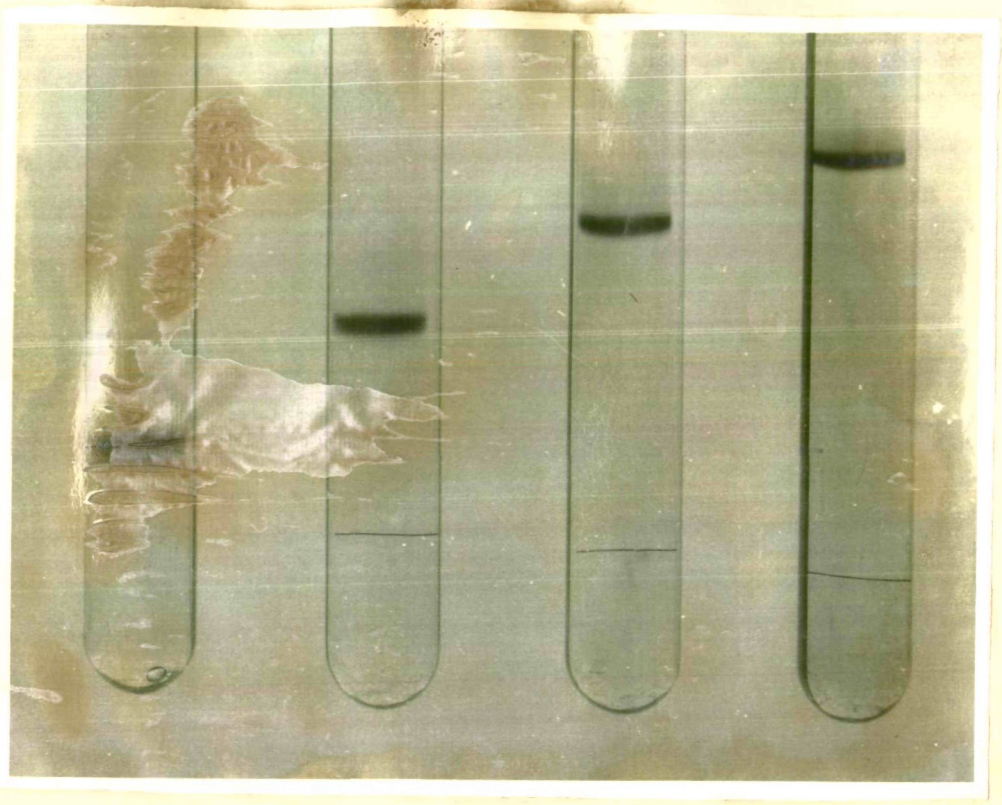
FIG. 6 ULTRACENTRIFUGE SCHLIEREN PHOTOGRAPHS AT THE MENISCUS FOR NITRITE REDUCTASE.

Buffer, 50 mM phosphate, pH 6.8; protein concentration, 7 mg/ml; speed, 8,210 rpm; temperature, 3.7°C; phase plate, 80°.
The determination of initial concentration (C_o) by layering solvent over protein solution made from a sequence of photographs like the one shown in Fig. 6 a.

A value of 0.73 ml/g for $\bar{v}$, calculated from the amino acid composition (ref. chapter V), was used. Mw determinations were carried out in 50 mM potassium phosphate buffer, pH 6.8, at three different protein concentrations in the range of 5-10 mg/ml. The value of Mw was found to be independent of protein concentration in the range tested. An average Mw value of 80,600 determined in the present work is significantly lower than the value of 95,000 reported by Prakash and Sadana (170) for ultracentrifugally pure enzyme. The discrepancy between the present value and that reported previously may be explained on the basis of inhomogeneity of the preparation used in the earlier work. The present value of Mw has been confirmed by several different methods. The results of determinations of the native enzyme by the Archibald procedure are presented in Table 8.

ii) <u>By disc gel electrophoresis</u>

The molecular weight was determined by analytical polyacrylamide gel electrophoresis according to the method of Hedrick and Smith (276). Nitrite reductase and the protein markers were subjected to electrophoresis in a series of gels which varied in acrylamide content from 6-12%; all other conditions of electrophoresis remained unchanged (ref. chapter II). Fig. 7 shows the electrophoretic behaviour of nitrite reductase in gels of varying acrylamide content. Under such conditions proteins migrate into the gel as a function of their size and charge and the acrylamide concentration in the gel. A plot of



% Gel 6 8 10 12

FIG. 7 DETERMINATION OF THE MOLECULAR WEIGHT OF NATIVE ENZYME BY DISC GEL ELECTROPHORESIS (SLOPE METHOD).

Enzyme samples (25-30 ug) in 50 ul of 6 mM tris and 47 mM glycine buffer pH 8.3 containing 50% glycerol and 0.05% bromophenol blue were loaded on top of the gels. Electrophoresis was carried out at 4 mA/gel for 2 hr at 4°C.

TABLE 8

MOLECULAR WEIGHT OF *A. FISCHERI* NITRITE REDUCTASE BY THE ARCHIBALD PROCEDURE[a]

Buffer system = potassium phosphate, pH 6.8, 0.05 M

Temperature = 3-6°C

Partial specific volume = 0.73 ml/g

Molecular weight calculated from readings at the meniscus

Protein concentration (mg/ml)	Rotor speed (r.p.m.)	Molecular weight
5.4	8,210	77,600
		83,700
		80,100
7.2	8,210	80,000
		79,200
		82,800
9.6	7,250	78,000
		81,200
		82,700
5-10		80,600 ± 2,000

[a]This method was preferred over that of Yphantis (269) because of the difficulty in obtaining a stabilized supply of current over a long period of time.

log relative mobility [100log (100 Rm)] versus gel concentration results in a straight line and a linear relationship exists between the slope of such a plot and the molecular weight of the protein. Results with nitrite reductase and markers are presented in Fig. 8(a) and 8(b). The molecular weight of nitrite reductase by this method was found to be 80,000 which agrees excellently with those obtained by other methods.

iii) By gel filtration

When the enzyme was chromatographed on Sephadex G-200 column with marker proteins of known molecular weight, the elution profile shown in Fig. 9 was obtained. The gel filtration data of the enzyme and the marker proteins in terms of K_d, K_{av} and V_e/V_o are presented in Table 9. The elution volumes were found to be reproducible. The elution position of nitrite reductase essentially coincides with that of bovine serum albumin. A plot of V_e/V_o versus logarithm of molecular weight (Fig. 10) according to the procedure of Andrews (270) gave a straight line and indicated that the molecular weight of the enzyme was 66,000-67,000 daltons. This value is smaller than the molecular weight estimated by other methods. To substantiate that the low molecular weight calculated according to the Andrews' procedure was not due to interactions between the enzyme and Sephadex polysaccharide matrix, the molecular weight was also redetermined by gel filtration technique using Bio-gel P-150, an inert polyacrylamide gel. In these experiments, all procedures were identical with those described

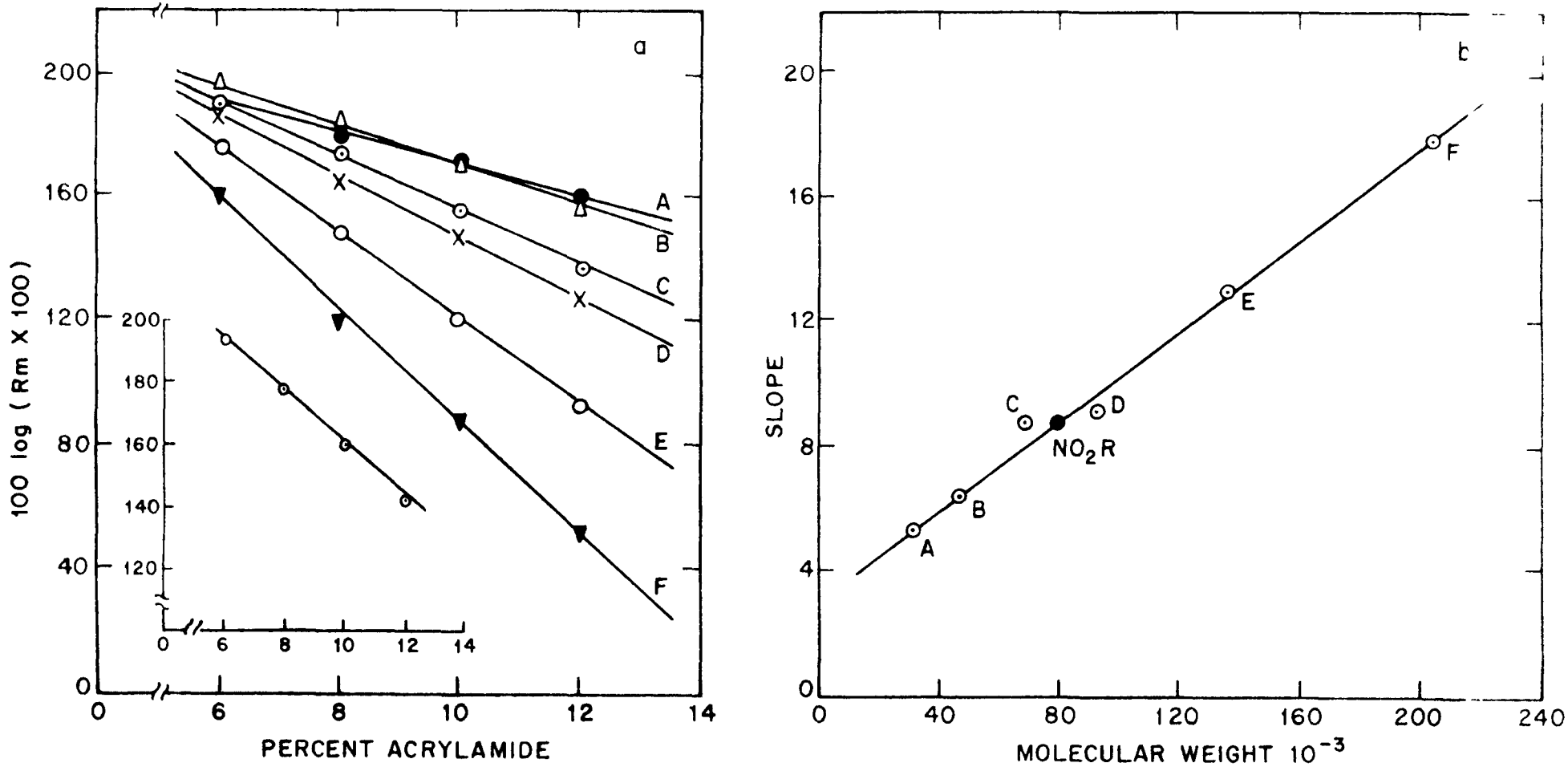


FIG. 8 DETERMINATION OF MOLECULAR WEIGHT OF A FISCHERI NITRITE REDUCTASE BY GEL ELECTROPHORESIS (SLOPE METHOD)

FIG. 8. DETERMINATION OF THE MOLECULAR WEIGHT OF A. FISCHERI NITRITE REDUCTASE BY GEL ELECTROPHORESIS (SLOPE METHOD)

Electrophoresis was carried out in the cold room at 4°C at 4 mA per tube for 2 hr. The ratio (R_m) of the migration of the protein band to that of bromophenol blue for 6,8,10 and 12% gel concentrations were determined. The ratio of acrylamide to methylenebisacrylamide was kept constant at 30:1. Migration of bromophenol blue and protein bands were measured on a illuminated box using a magnifying glass. Measurements were accurate to ± 0.5 mm.

(a) Plots of log R_m of protein markers versus gel concentration. Straight lines were obtained. The standard proteins used are: (A) deoxyribonuclease I, 31,000 (303); (B) ovalbumin, 46,000 (304); (C) bovine serum albumin, 68,000 (305); ovalbumin dimer, 92,000; (E) bovine serum albumin dimer, 136,000; (F) bovine serum albumin trimer, 204,000. In the case of deoxyribonuclease I the position of the major band was taken as representing the behaviour of the protein. In the insert is the plot of R_m of nitrite reductase versus gel concentration.

(b) The negative slope of each protein from Fig. 8a was plotted against the molecular weight. A straignt line was obtained. The molecular weight of nitrite reductase, computed from its slope on the calibration curve was, 80,000.

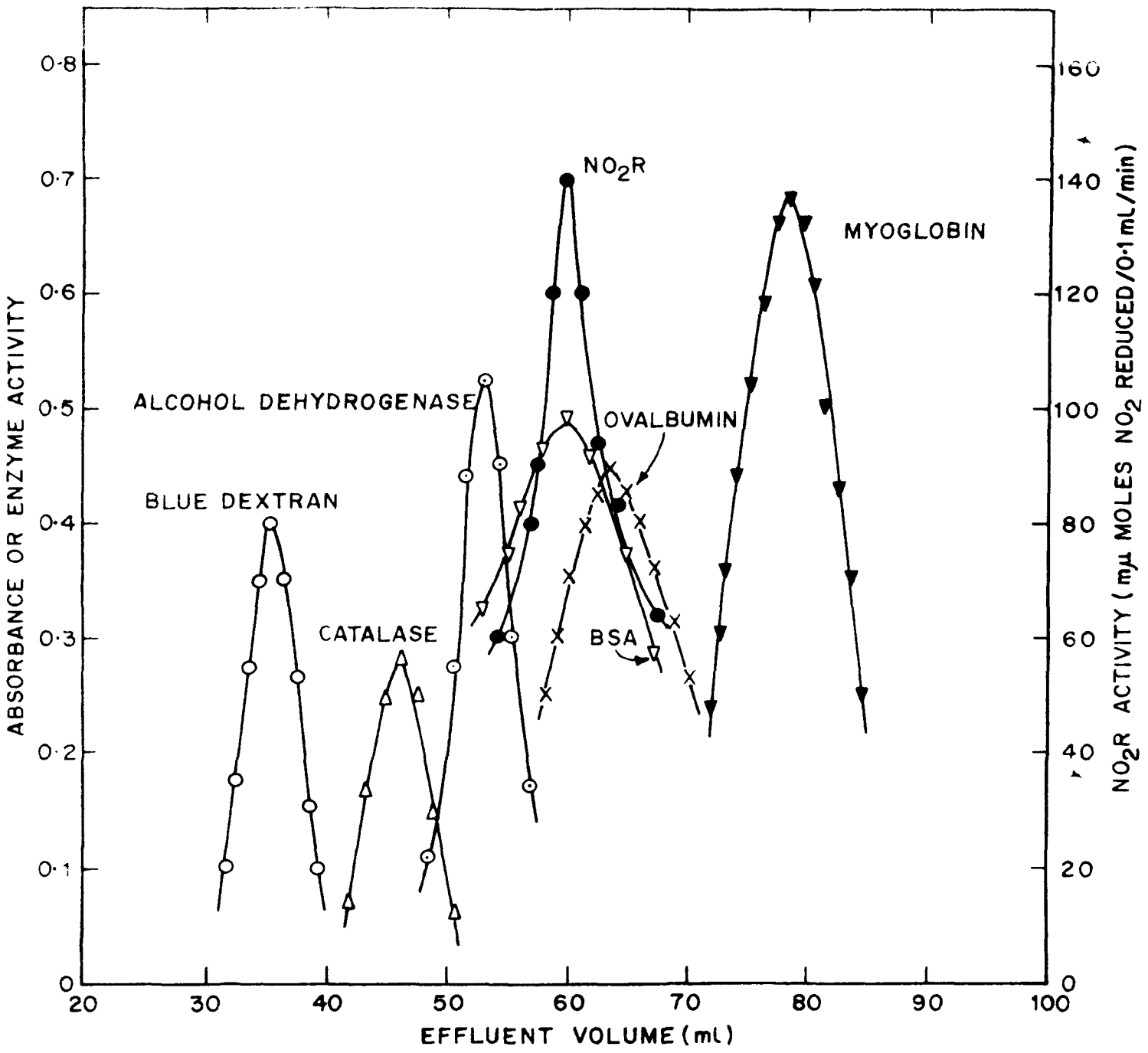


FIG. 9 CHROMATOGRAPHY OF A. FISCHERI NITRITE REDUCTASE ON SEPHADEX G. 200.

FIG. 9. CHROMATOGRAPHY OF A. FISCHERI NITRITE REDUCTASE ON SEPHADEX G-200

200 μg of nitrite reductase (specific activity = 1500) were applied on a Sephadex G-200 column (1.6 x 55 cm) along with 3 mg of Blue Dextran, 2 mg of catalase, 2 mg of alcohol dehydrogenase, 5 mg of bovine serum albumin and 2 mg of myoglobin in a final volume of 1.0 ml. Ovalbumin (5 mg in 0.5 ml) was chromatographed in a separate run with Blue Dextran. The column was equilibrated with 50 mM potassium phosphate buffer, pH 6.8. Elution was performed with the same buffer, and fractions of about 1.0 ml each were collected. Bovine serum albumin and ovalbumin were determined by absorption at 280 nm while myoglobin at 40.9 nm. Enzyme activities were assayed as described in the text: catalase (A_{240}/min per 40 μl); alcohol dehydrogenase (A_{340}/min per 0.1 mø1); nitrite reductase (mμmoles NO_2^- reduced per 0.1 ml per min). Blue dextran was measured at 625 nm.

TABLE 9 .. GEL FILTRATION DATA OF A. FISCHERI NITRITE REDUCTASE AND STANDARD PROTEINS

References to Stokes' radii are cited after protein listed. Stokes' radius of myoglobin was calculated from the diffusion coefficient. The K_d and K_{av} were calculated according to Siegel and Monty (274). The void volume of the column was 35.5 ml. Two separate gel filtration experiments were performed in order to determine the effective pore radius r of the bath of Sephadex G-200 used (271)

Proteins	Stokes' radii nm	Elution volume ml	$\frac{V_e}{V_o}$	K_d	K_{av}	r Calculated nm	r Average
Blue dextran 2000	-	35.5	-	-	-	-	-
Catalase (306)	5.23	46.2	1.30	0.170	0.166	16.0	
Alcohol dehydrogenase(274)	4.60	53.2	1.50	0.282	0.274	18.3	
Bovine serum albumin (306)	3.61	60.0	1.69	0.390	0.380	18.4	18.2
Ovalbumin (307)	2.76	63.8	1.80	0.451	0.439	16.3	
Myoglobin (308)	1.90	78.5	2.21	0.685	0.667	21.9	
A. fischeri nitrite reductase	-	60.0	1.69	0.390	0.380	-	

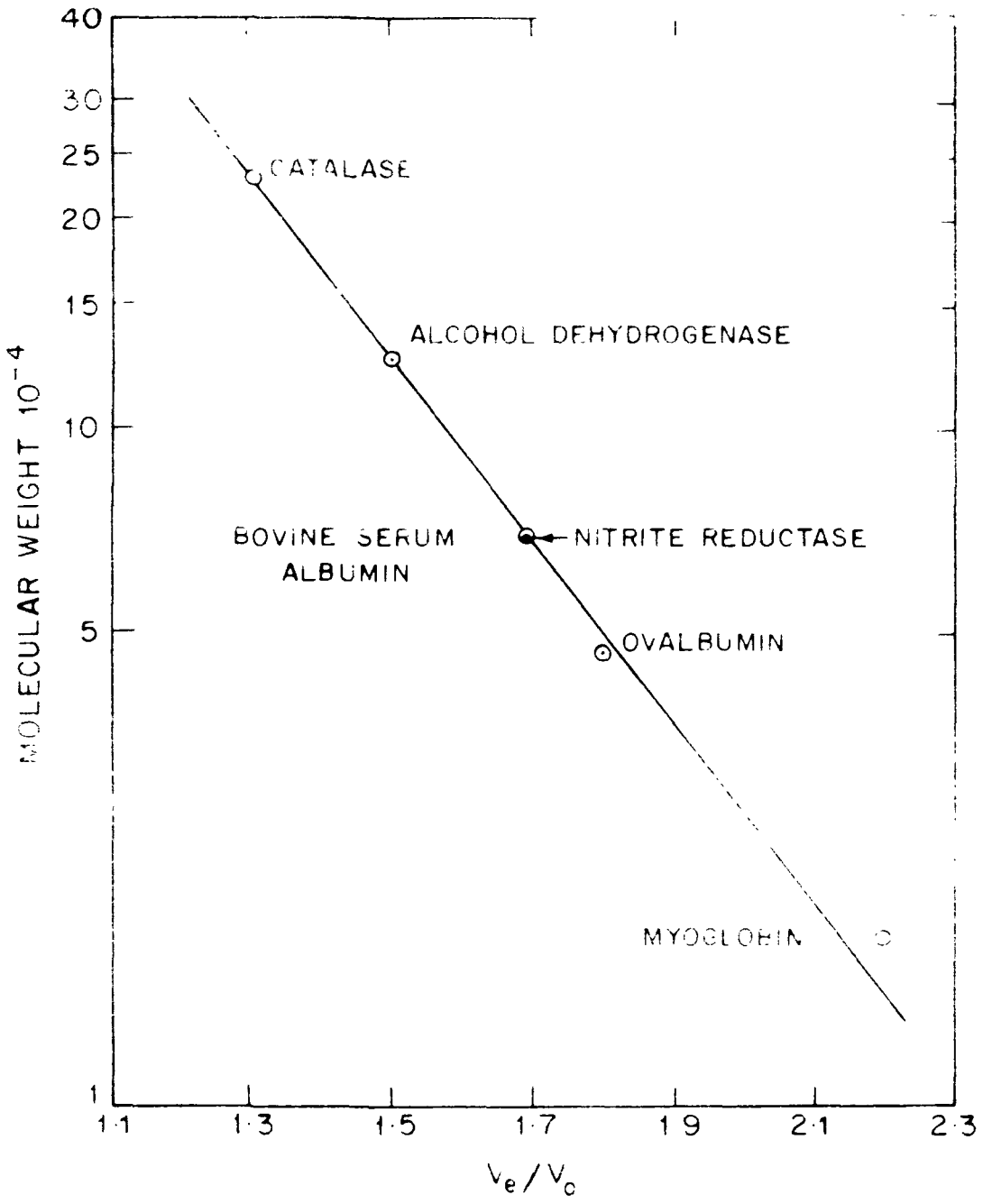


FIG. 10 DETERMINATION OF MOLECULAR WEIGHT OF A. FISCHERI NITRITE REDUCTASE BY GEL FILTRATION ON SEPHADEX G-200.
The elution data of Table 9 were employed The protein markers used were: catalase, 230,000(309); alcohol dehydrogenase, 125,000 (309); bovine serum albumin, 68,000(305); ovalbumin, 46,000 (304); and myoglobin, 17,600 (304). V_e/V_O values were plotted against log molecular weights according to the procedure of Andrews (270).

for Sephadex (ref. chapter II). The values of the molecular weight determined from calibrated Bio-gel P-150 column agree within experimental error, with those obtained using Sephadex G-200.

The behaviour of a protein in gel filtration is a function of its Stokes' radius rather than the molecular weight (271). The Stokes' radius for nitrite reductase was calculated from its distribution coefficients, K_d and K_{av} and the pore radius, r, of the column. A pore radius of 18.2 nm for the bath of Sephadex G-200 used was calculated from the known Stokes' radii of protein markers (271). A linear relationship (Fig. 11a and 11b) was obtained when the experimental data are plotted according to Porath (272), and Laurent and Killander (273). The validity of the relationship proposed by Ackers (271) is evident from the agreement obtained for the value of r using different standard proteins. The Stokes' radius of nitrite reductase was calculated by the methods of Ackers, Porath and Laurent and Killander. The three methods yield similar values (3.56, 3.47 and 3.45 nm) with an average of 3.49 nm.

Combination of this value with the sedimentation coefficient obtained from the ultracentrifuge experiments allows calculation of the molecular weight through application of the combined Stokes-Einstein and Svedberg equations (274):

$$M = \frac{6\pi N\eta\, a\, s^{\circ}_{20,w}}{(1 - \nabla\rho)} \qquad \ldots\ldots\ldots \quad (1)$$

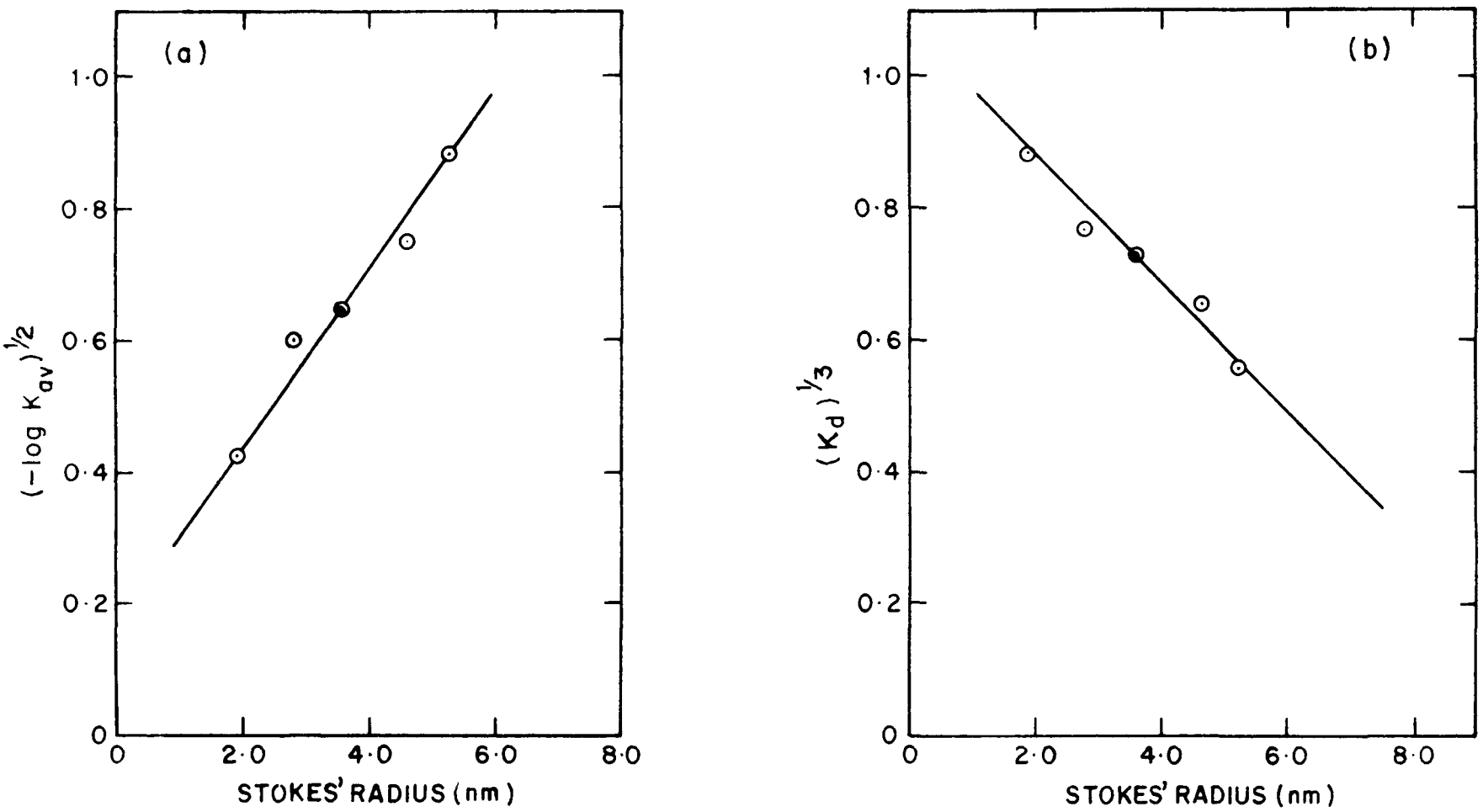


FIG. 11 ESTIMATION OF THE STOKES' RADIUS OF A. FISCHERI NITRITE REDUCTASE. The elution data of Table 9 were used. a) The data are plotted according to the correlation of Laurent and Killander (273). b) The data are plotted according to the correlation of Porath (272).

in which N is the Avogadro's number, η and n are the viscosity and density respectively of water at 20°C, a is the Stokes' radius, $\bar{V}$ the partial specific volume, and $s^{\circ}_{20,w}$ the sedimentation coefficient corrected to water at 20°C and extrapolated to zero protein concentration. Substitution of 0.73 ml/g for $\bar{V}$, determined from amino acid composition, (ref. Chapter V) and 5.25 S for $s^{\circ}_{20,w}$, yielded a molecular weight of 78,000. Although this value is slightly lower than that of 80,600 and 80,000 obtained ultracentrifugally and by the disc gel electrophoresis procedure respectively, it is within the precision expected for the gel filtration technique.

The diffusion coefficient could be calculated from Stokes' radius by the use of the Stokes-Einstein equation (274):

$$D_{20,w} = K\,T/6\pi\eta a \qquad \ldots\ldots\ldots \quad (2)$$

where K is the Boltzman constant, T is the absolute temperature and η and a have the same meaning as in equation 1. The diffusion coefficient ($D_{20,w}$) of nitrite reductase calculated from the gel filtration data (a = 3.49 nm) is 6.05F. The diffusion coefficient could be combined with the sedimentation coefficient to calculate the molecular weight by the use of the Svedberg equation (310):

$$M = \frac{s^{\circ}_{20,w}\,R\,T}{D_{20,w}\,(1 - \bar{V})} \qquad \ldots\ldots\ldots \quad (3)$$

where R is the gas constant and $\bar{V}$ is the partial specific volume. All other symbols have the same meaning as described in equations 1 and 2. A molecular weight of 79,600 is obtained

which agrees with the ultracentrifugal value.

FRICTIONAL RATIO AND AXIAL RATIO OF THE NATIVE ENZYME:

The frictional ratio, f/f_o, can be calculated from Stokes' radius according to the following equation (274):

$$f/f_o = \frac{a}{\left(\frac{3M\bar{V}}{4\pi N}\right)^{-1/3}} \qquad \ldots\ldots\ldots \quad (4)$$

Substituting Stokes' radius and a molecular weight value of 80,000 in equation 4, a value of 1.25 was obtained for the frictional ratio of nitrite reductase. An identical value of frictional ratio was calculated from the ultracentrifugal data by the following equations (311):

$$f = \frac{M(1 - \bar{v})}{N\, s^{o}_{20,w}} \qquad \ldots\ldots\ldots \quad (5)$$

$$f_o = \eta \left(\frac{162\, M\, \bar{v}}{N}\right)^{1/3} \qquad \ldots\ldots\ldots \quad (6)$$

All the symbols in equations 4,5 and 6 have the same meaning as above.

Asphericity and solvation in a molecule result in a frictional ratio greater than unity. Assuming the average solvation. (0.3 g/g protein), a value of 1.25 for f/f_o would yield an axial ratio of 3 for A. fischeri nitrite reductase (312).

SUBUNIT STRUCTURE

i) By ultracentrifugation:

The average molecular weight value of 78,700 and 79,800 obtained in 6M urea and 6M Gu.HCl in the absence of 2-ME are

very close to that determined for the native enzyme indicating that the enzyme does not dissociate on treatment with these denaturing agents in the absence of 2-ME. The sedimentation coefficient of the enzyme under such conditions was also similar to that of the untreated enzyme. The addition of 1% 2-ME to the solution of the enzyme in 6M Gu.HCl resulted in a marked reduction in the sedimentation coefficient (1.4 S). A molecular weight of 39,000 for nitrite reductase was obtained in 6M Gu.HCl containing 1% 2-ME indicating the presence of two subunits. This dissociation which occurs in the presence of a reducing agent, indicates that disulfide bond(s) may be involved in the binding of polypeptide chains of nitrite reductase. The presence of a single symmetrical peak under dissociating conditions suggests that the subunits are apparently of identical size and that the dissociation is complete. Gu.HCl was chosen for dissociation studies for its stronger denaturing action as compared to urea. The results of the molecular weight determinations (Archibald procedure) in 6M urea and 6M Gu.HCl with and without reducing agent are presented in Table 10. The molecular weight of the native enzyme is included in Table 10 for the sake of comparison. The values for molecular weight of the denatured enzyme are based upon the assumption that $\bar{v}$ in the denaturing solvents decreases by 0.01 ml/g (313-315).

ii) By SDS-gel electrophoresis:

The molecular weight of the nitrite reductase subunit was also determined by its migration in SDS-gels according to

TABLE 10

SEDIMENTATION DATA ON *ACHROMOBACTER* NITRITE REDUCTASE AND ITS SUBUNITS

Buffer = Potassium Phosphate Temperature = 20-25°C

Rotor speed = 14,290 - 17,250 rpm

$\bar{v}$ = 0.73

Solvent system	Protein (mg/ml)	Molecular Weight	$s_{20,w}$ (S)
1. 50 mM buffer, pH 6.8	5-10	80,600 ± 2,000	5.25 ($s^{\circ}_{20,w}$)
2. 6 M Urea - 0.15 M NaCl 2mM EDTA, 50 mM buffer pH 7.0	9.0	80,300 79,000 77,000	5.14
	Average	78,700	
3. 6M Gu.HCl - 0.15 M NaCl 2 mM EDTA - 50 mM buffer pH 7.0	8.5	81,500 80,200 77,800	
	Average	79,800	
4. 6M Gu.HCl - 0.1 M 2-ME- 0.15 M NaCl - 2mM EDTA -50 mM buffer pH 7.0	7.0	40,100 37,600 39,400	1.40
	Average	39,000	

the method of Shapiro et al. (278). The enzyme and the marker proteins were incubated for 4 hr at 37°C with 1% SDS and 1% 2-ME before subjecting them to SDS gel electrophoresis. Semilog plot of molecular weight verus relative mobility of the marker proteins yielded a straight line (Fig. 12) and an estimate of the molecular weight of the enzyme subunits of 38,000. A small amount of the protein migrates corresponding to a molecular weight of 78,000 which represents the undissociated enzyme. This has been observed in the case of several enzymes having quaternary structure (316,317). Only one protein band was detectable for nitrite reductase subunits in SDS-gel electrophoresis indicating that the two subunits in the native enzyme are very similar if not identical.

The physical parameters of nitrite reductase are summarized in Table 11.

PRESENCE OF DISULFIDE BONDS:

Titration of nitrite reductase in the absence or presence of 8M urea or 8M urea plus 1% SDS by DTNB or p-HMB yields four sulfhydryl groups per enzyme molecule. The titration of the total number of thiol groups by DTNB after borohydride reduction in the presence of 8M urea, (which is the sum of free thiol groups and the ones generated by reduction of disulfide bond(s)), gave six thiol groups. These results suggest that nitrite reductase contains four free sulfhydryl groups and one disulfide bond per mole of native

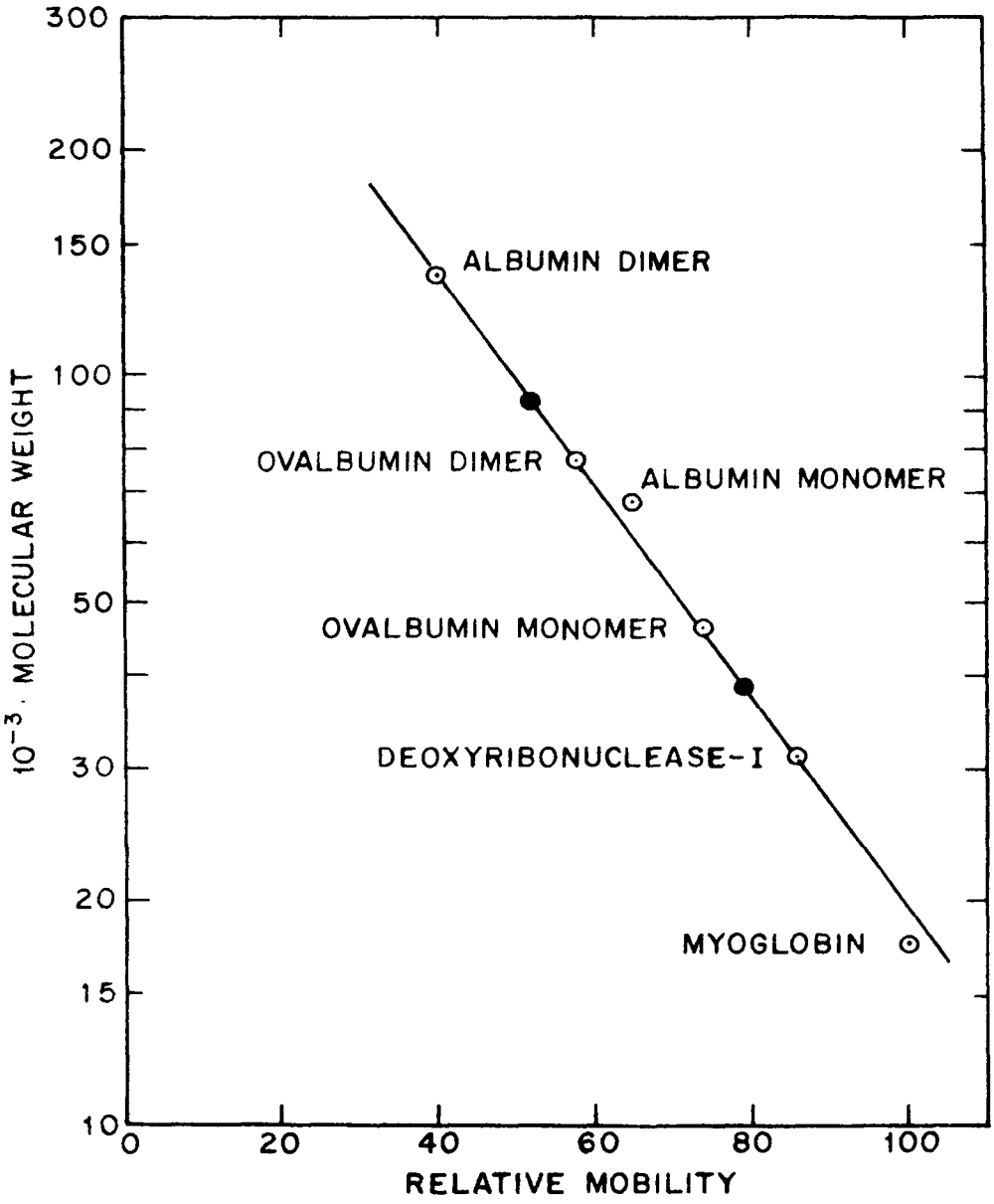


FIG. 12 MOLECULAR WEIGHT ESTIMATION OF A. FISCHERI NITRITE REDUCTASE SUBUNITS BY SDS-GEL ELECTROPHORESIS. The enzyme (25-30 μg) was incubated with 1% SDS and 1% 2-ME at 37 °C for 4 hr. Electrophoresis was carried out at 28-30 °C for 3 hr at 8 mA per tube. The nitrite reductase gave two bands (●), a major band corresponding to 38,000 molecular weight and a faint band corresponding to a molecular weight of 78,000.

TABLE 4

SUMMARY OF PHYSICO-CHEMICAL PROPERTIES OF *A. FISCHERI* NITRITE REDUCTASE

Parameter	Determination	Value
Stokes' radius	Ackers (271)	3.56 nm
	Porath (272)	3.47 nm
	Laurent and Killander (273)	3.45 nm
	Average	3.49 nm
Diffusion coefficient, $D_{20,w}$	from Stokes' radius (274)	6,05 $cm^2.sec^{-1}$
Sedimentation coefficient, $s^{\circ}_{20,w}$	(264)	5,25 S
Molecular weight:		
(a) Native enzyme:	from ultracentrifuge (265)	80600
	from Stokes' radius and sedimentation coefficient (274)	78200
	from diffusion coefficient and sedimentation coefficient (310)	79600
	from polyacrylamide gel electrophoreis (276)	80000
	from gel chromatography by Andrews procedure on Sephadex G-200 or Bio-gel P-150 (270)	66000-67000
(b) Subunits:	from ultracentrifuge (265)	39000
	from SDS-gel electrophoresis (278)	38000
Frictional ratio	(274,311)	1.25
Axial ratio	~~(274,311)~~(312)	3.0
N-Terminal amino acid	(279)	Methionine

enzyme. The results of half-cystine and free thiol groups estimations are presented and further discussed in chapter V.

N-TERMINAL ANALYSIS

Preliminary experiments with dansyl-nitrite reductase using solvent system C of Morse and Horecker (280) indicated that methionine was the N-terminal residue. Under these conditions dansylalanine and dansylphenylalanine were the only other two amino acids which came close to dansylmethionine on thin layer chromatograms. Chromatography of dansylphenylalanine, dansylalanine and dansylmethionine in the solvent system A of Morse and Horecker (280) and solvent system b of Deyl and Rosmus (281) gave unambiguous separations. One-dimensional thin layer chromatography of hydrolyzed dansyl-enzyme using the solvent A of Morse and Horecker (Fig. 13) and system b of Deyl and Rosmus yields one spot corresponding to dansylmethionine. Only one spot was obtained when the sample was co-chromatographed with authentic dansylmethionine (Sigma) added as the internal standard. These experiments indicated methionine as the only N-terminal residue. The failure to find any N-terminal amino acid other than methionine suggests that the two subunits are similar though not necessarily identical.

Quantitative determination of N-terminal amino acid was undertaken in the hope of obtaining additional evidence on the number of peptide chains in the native nitrite reductase. The dansylation of the enzyme with 20 mM dansyl chloride in 4 M urea under the experimental conditions described in Materials

1 2 3 4 5 6

FIG. 13 DETERMINATION OF N-TERMINAL AMINO ACID OF THE ENZYME.

The dansylated amino acids were separated by thin layer chromatography on silica gel by using the solvent system A of Morse and Horecker (280), benzene : pyridine : acetic acid (80 : 20 : 2, v/v/v). 1= dansylphenylalanine, 2= dansylmethionine, 3= mixture of dansylphenylalanine, dansylmethionine and dansylalanine, 4= dansyl-enzyme, 5= dansylalanine and 6= dansyl-NH_2. The N-terminal was methionine.

and Methods gave yields of 1.2 and 1.3 mole of dansylmethionine per mole of the enzyme (after correction for losses during hydrolysis and isolation procedure was applied). Control experiments with methionine gave dansylmethionine in about 50-60% of the theoretical yield which is very close to that reported by Gros and Labouesse (279) for methionine. Although the yields of N-terminal are low, the results support the dimeric structure of A. fischeri nitrite reductase.

DISCUSSION

Although bacterial nitrite reductases have been studied in a variety of organisms, none has been obtained in a homogeneous state to permit a detailed characterization. The present work on A. fischeri enzyme forms the first detailed characterization of nitrite reductase which is homogeneous both in the ultracentrifuge as well as in polyacrylamide disc gel electrophoresis.

Results of sedimentation velocity studies at protein concentrations in the range of 1-9 mg/ml gave an $s^{o}_{20,w}$ of 5.25 S with a slight dependence on protein concentration. The diffusion coefficient determined from the gel filtration experiments with Sephadex G-200 is 6.05 F. This value is close to that reported for P. aeruginosa and spinach enzymes.

An average molecular weight of 80,000 for A. fischeri nitrite reductase was determined in the present work. Several independent methods viz. Archibald approach-to-equilibrium method,

from sedimentation and diffusion coefficients, from sedimentation coefficients and Stokes' radius, and analytical disc gel electrophoresis procedure of Hedrick and Smith (276), were used and values of molecular weights obtained were in good agreement. The present value of 80,000 is smaller than that previously reported for the ultracentrifugally (but not electrophoretically) pure enzyme. It seems likely that the earlier value was an average of the molecular weights of the enzyme and the impurities.

The molecular weights reported for nitrite reductase from algae and higher plants fall within the narrow range of 60,000-72,000. In contrast to the enzyme from green tissues, the bacterial nitrite reductases exhibit a greater degree of variation. Molecular weight as low as 67,000 has been reported for nitrite reductase from A. chroococcum (156) and as high as 200,000 for the enzyme from P. perfectomarinus (3). Though sedimentation studies have also been carried out, in most cases the molecular weight values were determined by gel chromatography on Sephadex G-200.

Squire (318) and Ackers (271) have provided a theoretical basis for the physico-chemical characterization of proteins by Spphadex gel filtration. Although the mechanism of gel filtration has not been completely explained, the correlation between elution volume and molecular weight holds true for a great number of proteins and enzymes. Exceptions are exemplified by non-globular proteins and also by some glycoproteins. Siegel and Monty (274) ~~provided~~ presented evidence which strongly indicated that

the elution position of a protein upon Sephadex G-200 chromatography is not correlated with molecular weight but instead is a function of the Stokes' radius. The molecular weight of 66,000-67,000 determined for A. fischeri nitrite reductase by the Andrews' procedure in the present studies is significantly low and is not consistent with the values estimated in the ultracentrifuge and disc gel electrophoresis. However, when Stokes' radius, determined from the same gel filtration data, was combined with the sedimentation coefficient, a molecular weight of 78,000 was obtained. The agreement between this value and that of 80,000 obtained by other methods further supports the usefulness of correlating the behaviour of proteins on gel filtration with their Stokes' radii. It is of interest to note that gel filtration is reported to yield a considerably lower value of 60,000 for spinach nitrite reductase as compared to that of 72,000 determined from the sedimentation data (164). However, no attempt was made to correlate the elution volume with Stokes' radius.

The number of proteins which are known to form stable conformation by the assembly of discrete subunits is now very large as witnessed by a recent review article of Irwing et al.(319)

It has been suggested that proteins whose molecular weights are over 50,000 may be expected to reveal such a subunit structure (320). In most cases, the bonding is that of the noncovalent linkages, although some proteins have been found to be made of several polypeptide chains covalently linked by disulfide bridges. Nitrite reductase from A. fischeri does not dissociate when treated with 6M urea or 6M Gu.HCl indicating either very strong interactions between the subunits or covalent linkages. However, in an adequate reducing environment 6M Gu.HCl or 1% SDS dissociate A. fischeri nitrite reductase into its polypeptide chains. A molecular weight of 39,000 for the reduced enzyme in 6M Gu.HCl was determined by the Archibald procedure suggesting that the native enzyme is composed of two polypeptide chains which are linked together by disulfide bond(s).

Additional evidence for the dimeric structure of A. fischeri nitrite reductase is derived from the results of SDS-polyacrylamide gel electrophoresis. A subunit molecular weight of 38,000 was estimated by electrophoresis in SDS-polyacrylamide gels.

Titration of the enzyme with DTNB or p-HMB in denaturing medium for thiol groups before and after borohydride reduction indicated the presence of one disulfide bond. Several lines of evidence indicate that the subunits are highly similar. Thus, under dissociating conditions (6M Gu.HCl-0.1M 2-ME) a single, symmetrical peak was observed upon sedimentation in the ultracentrifuge. Similarly, there was no detectable difference

in the molecular weight upon SDS-polyacrylamide disc gel electrophoreis for only one protein band was detected for the nym enzyme subunits. Furthermore, methionine was the only N-terminal amino acid determined by dansylation studies. The quantitative determination of N-terminal amino acid gave a value of 1.2 - 1.3 mole of methionine per mole of enzyme. Although the results are not conclusive, they support the dimeric structure of A. fischeri nitrite reductase. The low yield, though attributable to incomplete dansylation and losses during hydrolysis and isolation procedure, leaves open the possibility of other N-terminal groups. Low recoveries of N-terminal amino acid have been reported for other proteins also. Thus dansylation (50 mM dansyl chloride) of bacitracin, glutamate dehydrogenase, and β-lactoglobulin gave yields of N-terminal of 51,40 and 62% of the theoretical value (after the correction was applied) (321).

Data obtained permit the inference that A. fischeri nitrite reductase consists of two similar, though not necessarily identical, polypeptide chains which are covalently bonded by a disulfide bridge and are of the same size.

Though there has been no detailed study, evidence in support of the subunit structure in nitrite reductases from spinach (164) and P. aeruginosa (167) has been obtained. The native spinach nitrite reductase has a molecular weight of 72,000 as determined by the sedimentation data. The results of SDS-polyacrylamide gel electrophoresis suggested that the native enzyme consists of two subunits of molecular weight of

37,000. The subunit structure of spinach nitrite reductase resembles that of A. fischeri enzyme reported here. Similarly, the sucrose density centrifugation of SDS-treated P. aeruginosa nitrite reductase suggested that the native enzyme which has a molecular of 67,000 consists of two subunits. However, the enzyme from C. fusca (135) has been reported to consists of one polypeptide chain.

CHAPTER 5

AMINO ACID COMPOSITION AND HYDRODYNAMIC PROPERTIES

SUMMARY

The amino acid composition of *A. fischeri* nitrite reductase was analyzed. The analyses indicated an amino acid composition for a total of 693 amino acid residues of: Trp_8, 1/2-Cys_{10}, Met_{17}, Arg_{18}, His_{22}, Pro_{24}, Tyr_{26}, Phe_{34}, Val_{41}, Gly_{42}, Ser_{42}, Ile_{44}, Thr_{44}, Leu_{48}, Lys_{50}, Ala_{52}, Asp_{85}, Glu_{86}, and amide amonia$_{62}$. Independent determinations of tyrosine and tryptophan were in good agreement with each other and with the tyrosine content determined in the Amino acid analyzer. In the absence as well as in the presence of denaturants, four sulfhydryl groups react with p-hydroxymercuribenzoate (p-HMB), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Reduction of the enzyme with borohydride followed by titration with DTNB gave six -SH groups indicating the presence of one disulfide bond in the enzyme molecule. Titration of all the four sulfhydryl groups does not inactivate the enzyme. Inactivation of the enzyme with p-HMB and p-chloromercuribenzene sulfonic acid at relatively higher concentrations is not related to mercaptide formation.

The isoionic point and the partial specific volume were determined to be 5.1 and 7.3 ml/g from the amino acid composition. The hydrophobicities of the enzyme computed from the amino acid composition in terms of the average hydrophobicity ($H\phi_{av}$), polarity index (p), and the frequency of non-polar side chains (NPS) were found to be 1075 cal/residue, 1.00, and 0.32 respectively.

INTRODUCTION

A. fischeri nitrite reductase catalyzes the reduction of nitrite to ammonia. Hydroxylamine is not a free intermediate during this 6-electron reduction though the enzyme also reduces hydroxylamine to ammonia (170). Enzyme chemical studies will be required to elucidate the reaction mechanism by which the enzyme carries out the complex multielectron reduction of nitrite to ammonia. Such a study was made possible by the isolation of the homogeneous enzyme with high yield (ref. chapter III).

A quantitative amino acid analysis is the basis for any sequence studies and provides the foundation for a chemical evaluation of the enzyme reaction and the relationship between the chemical and physical properties of the protein. As a first step towards the chemical characterization of *Achromobacter* nitrite reductase, an analysis of the primary structure of the enzyme by determining its amino acid composition was undertaken. The results of this study form the subject matter of this chapter.

The amino acid composition of nitrite reductases from *P. aeruginosa* (167), and *C. fusca* (135) have been reported. The amino acid compositions of *A. fischeri* nitrite reductase and those from *P. aeruginosa*, and *C. fusca* are found to be quite similar when comparison is made on weight percent basis. The partial specific volume, the isoionic point and the degree of hydrophobicity of *A. fischeri* ~~of A. fischeri~~ nitrite reductase calculated from the amino acid composition are also reported.

Nitrite reductases from several sources have been shown to be sensitive to p-HMB and a number of other thiol-reactive compounds (82,114,137,160,170-172). The inhibition by p-HMB is reversible in most cases when incubated with excess of cysteine or reduced glutathione (114,137,145,170,171). The possible site of action of thiol-reactive compounds is assumed to be the sulfhydryls but no titration studies with purified enzyme were reported. In a preliminary report from this laboratory, Prakash and Sadana (170) showed that at relatively higher concentrations, p-HMB and p-CMS produced inactivation of A. fischeri nitrite reductase. The inhibition by p-HMB and p-CMS was completely reversed by subsequent incubation with SH-containing compounds, cysteine and reduced glutathione. The work presented in this chapter was also aimed at obtaining evidence on the contents of sulfhydryl and disulfide groups and the involvement of sulfhydryls in the activity of the enzyme.

The work presented in this chapter has already been published (Mazhar Husain and J.C. Sadana (1974) Arch. Biochem. Biophys. 163, 21-28).

RESULTS

Amino acid composition:

The amino acid analyses of nitrite reductase were performed on duplicate samples without prior removal of heme groups. Hydrolysis of the enzyme was carried out for 18,24,30 and 48 hours as described under Materials and Methods.

Table 12 summarizes the amino acid recoveries obtained from the hydrolysates after chromatography. The amino acid composition, calculated by determining the molar ratio of each amino acid with respect to alanine, according to the procedure of Markland and Damus (322) is presented in the last column of Table 12. The calculated number of residues per mole of nitrite reductase is based upon the molecular weight of 80,000 determined by the Archibald approach-to-equilibrium method and disc gel electrophoresis (ref. chapter IV). The calculated value of each amino acid except isoleucine, valine, serine, threonine, half-cystine, and tryptophan is the average of the values reported for each time of hydrolysis. The analysis shows a predominance of lysine, alanine, aspartic acid, and glutamic acid residues whereas those of tryptophan, half-cystine, methionine, and arginine are slow in number. The total number of amino acid residues per mole of nitrite reductase was determined to be 693 and a consequent molecular weight of 80,030. The nitrogen content calculated from the amino acid composition is 15.96% which is the average value reported for proteins. The amide content of the enzyme was determined from the amount of ammonia

TABLE 12

AMINO ACID COMPOSITION OF *A. FISCHERI* NITRITE REDUCTASE[a]

Amino acid	Amount of amino acid recovered (μmoles)[b]					Extrapolated or best average ratio to alanine	Composition (moles/ 80,000
	Hydrolysis time (hr)						
	18	24	30	48	Ox[c]		
Trp	-	-	-	-	-	-	8[e]
1/2-Cys	-	-	-	-	.0109	.207	10[f]
Met	0.0186	0.0163	.0195	.0190	.0176	.334	17[g]
Arg	.0188	.0207	.0217	.0218	.0202	.358	18
His	.0236	.0254	.0240	.0249	.004	.423	22
Pro	.0284	.0291	.0271	.0257	.0282	.475	24
Tyr	.0278	.0292	(.0271)	.0291	-	.501	26[e]
Phe	.0374	.0364	(.0354)	.0389	.0292	.656	34
Val	.0370	.0394	.0410	.0450	.0418	.800	41[h]
Gly	.0474	.0476	(.0645)	0.0456	.0458	.818	42
Ser	.0459	.0435	.0430	.0395	.0433	.820	42[i]
Ile	.0368	.0400	.0390	.0478	.0440	.848	44[h]
Thr	.0486	.0472	.0454	.0446	.0450	.850	44[i]
Leu	.0561	.0534	.0503	.0582	.0575	.940	48
Lys	.0541	.0561	.0553	.0574	.0614	.961	50
Ala	.0577	.0578	.0603	.0564	.0560	1.000	52
Asp	.0974	.0957	.0940	.0961	.0973	1.652	85
Glu	.0980	.0988	.0983	.0934	.0992	1.674	86
Amide-NH_3	.1150	.1410	.1550	-	-	1.190	62[i]

[a] Conditions are described in the text. Analyses were performed on duplicate samples. Numbers in parentheses were not included in calculation of the final value for that amino acid.

[b] In order to compare one analysis from another, each set of values was multiplied by a factor so that the sum of recoveries of all amino acids except Thr, Ser, Val, Ile and 1/2 Cys were constant.

[c] Analysis after performic acid oxidation. Values for only cysteine and methionine were used.

[d] Calculated according to Markland and Damus (322).

[e] Measured by spectrophotometric and colorimetric methods as described in the text.

[f] Determined as cysteic acid

[g] Determined as methionine sulfone

[h] 48-h

[i] Extr

liberated during acid hydrolysis of the enzyme. No separate amide determinations were performed because of insufficient amount of the enzyme.

The contents of sulfhydryl groups and disulfide bonds, tyrosine and tryptophan were determined by different methods as described below.

Determination of tyrosine and tryptophan

i) Goodwin and Morton's method (291):

The purified enzyme (0.4 mg) was taken in 1.0 ml of 0.1 M NaOH and the absorbance of the enzyme at 294.4 nm and 280 nm was recorded. The tyrosine and tryptophan contents were determined by Goodwin and Morton's method (ref. chapter II) after applying the correction for heme absorption. The correction applied for heme absorption was 0.1. From the optical densities at 294.4 nm (0.43) and 280 nm (0.44), it was calculated that nitrite reductase from A. fischeri contains 27 moles of tyrosine and 9 moles of tryptophan residues per mole of enzyme.

ii) Bencze and Schmid's method (290):

The absorbance of purified enzyme solution (0.4 mg/ml in 0.1 M NaOH) was measured between 272 and 294 nm at 2 nm intervals. A graph of absorbance vs wavelength was plotted (Fig. 14). The maximal absorbance (A_{max}) of the absorption curve (after heme correction, 0.1) was 0.515. Two maxima appeared at about 282 nm and 290 nm. A line was drawn tangential to these two characteristic peaks. The slope of the tangent indicated a tyrosine-tryptophan ratio of 3.0 and an E value

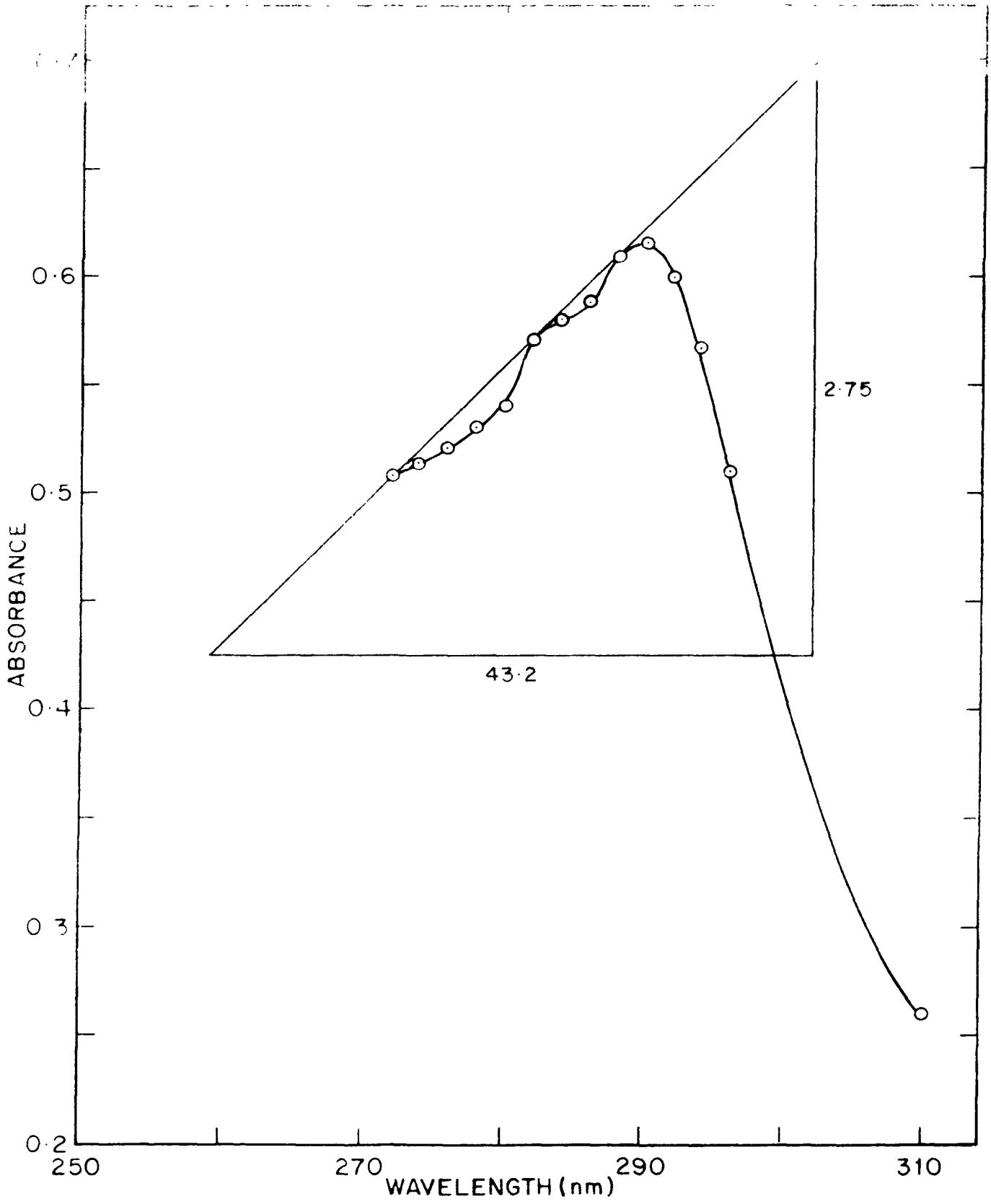


FIG 14 DETERMINATION OF TRYPTOPHAN AND TYROSINE CONTENTS OF A. FISCHERI NITRITE REDUCTASE BY BENCZE AND SCHMID'S METHOD. The absorbance of 0·4 mg per ml of the enzyme in 0·1 M NaOH was recorded between 272 nm and 296 nm at 2 nm intervals. The tyrosine and tryptophan content of the enzyme was determined according to Bencze and Schmid's method (290) as described in the text.

of 161. The tyrosine-tryptophan content was 0.515/161 = 3.2 mg per 100 ml of solution or 8% of the protein.

Assuming a molecular weight of 80,000 and a ratio of 3 to 1, nitrite reductase would contain 27 moles of tyrosine and 9 moles of tryptophan per mole of enzyme. These values are the same as that obtained from Goodwin and Morton's method.

(iii) Colorimetric methods:

The contents of tyrosine and tryptophan were also determined by colorimetric methods. Tyrosine was estimated on duplicate enzyme samples (0.57 and 1.14 mg) by the method of Uehara, Mannen and Kishida (292) with a standard solution of tyrosine (15 to 150 μg). A value of 25 tyrosine residues per mole of enzyme was obtained.

The tryptophan content of nitrite reductase (1.1 mg) was determined in duplicate by the colorimetric procedure of Spies and Chambers (293). A standard curve using various concentrations of tryptophan between 20 and 120 μg was constructed. A value of 7 tryptophan residues per mole of enzyme was indicated.

The results of tyrosine and tryptophan determinations are summarized in Table 13. These are in satisfactory agreement with each other and with the tyrosine content determined in the Amino acid analyzer. An average value of 8 tryptophan and 26 tyrosine residues per mole of enzyme is obtained.

Determination of free sulfhydryl groups:

The total number of free sulfhydryl residues per mole of enzyme was determined by spectrophotometric titration of the enzyme with p-HMB as described by Benesch and Benesch (284).

TABLE 13

TRYPTOPHAN AND TYROSINE DETERMINATION OF NITRITE REDUCTASE

Method	Tyr/Trp (mole/mole)	Residues/mole	
		Tyrosine	Tryptophan
Spectrophotometric			
i) Bencze and Schmid (290) (uv, 0.1 N NaOH)	3.0	27	9
ii) Goodwin and Morton (291) (uv, 0.1 N NaOH)	3.0	27	9
Colorimetric			
i) Spies and Chambers (293) (procedure K)	-	-	7
ii) Uehara et al.(292)	-	25	-
Amino acid analyzer	-	26	8[a]

[a]Calculated from the tyrosine content and a Tyr/Trp ratio of 3.0

Titration of the enzyme was carried out in its native form as well as after denaturation with 8 M urea, 1% SDS, and 8 M urea plus 1% SDS (ref. chapter II). Fig. 15 represents results which are typical of several experiments performed in the presence or absence of denaturing agents. From the data in Table 14, it is apparent that Achromobacter nitrite reductase contains four -SH group per mole of enzyme in the native and in the denatured state. In the absence of denaturing agents, however, the reaction proceeds at a slower rate taking a total time of 2-3 hr for completion whereas when the denaturants were included the reaction was complete within 20-40 min.

The free sulfhydryls of the nitrite reductase were also determined by Ellman's procedure with DTNB as described by Thorner and Paulus (285). The titration of the enzyme (0.2 - 0.4 mg) was performed in the absence and presence of denaturing agents (8 M urea or 1% SDS). The data in Fig. 16 indicate that in the native as well as in denatured state about four sulfhydryls per mole react with the Ellman's reagent. In the presence of denaturants the reaction was complete in an hour's time. In the absence of the denaturants, 2 sulfhydryls reacted fast (1 hr) while the remaining 2 -SH groups took about 5-6 hr, so that the total time taken for all the sulfhydryls to react in the absence of the denaturants was about 6-7 hr.

The finding of the same value in the presence or absence of denaturants indicates that all the free -SH groups are accessible for reaction with DTNB and p-HMB. The results of sulfhydryl determinations are summarized in Table 15.

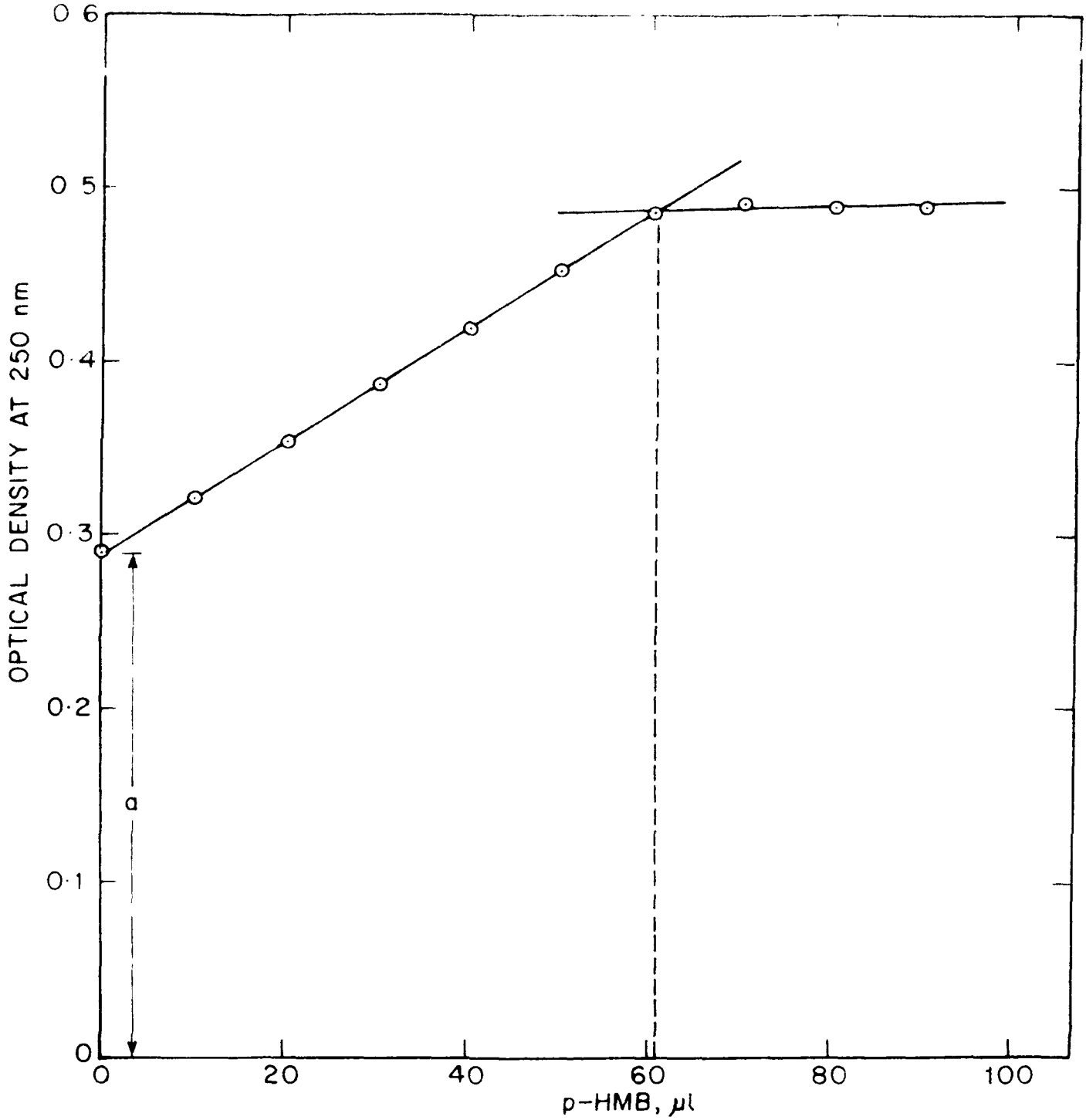


FIG. 15 DETERMINATION OF SULFHYDRYL GROUPS OF A. FISCHERI NITRITE REDUCTASE BY SPECTROPHOTOMETRIC TITRATION WITH p-HMB
Samples containing 0 43 mg of protein were taken in 1·0 ml of 0·05 M potassium phosphate buffer (pH 7 0) Aliquots of p-HMB ($3{\cdot}85 \times 10^{-4}$ M) were added to the blank cell containing the buffer and to the experimental cell containing protein and absorbancy at 250 nm was recorded. The intercept, a, is due to absorption of the protein

TABLE 14

SPECTROPHOTOMETRIC TITRATION OF A. FISCHERI NITRITE REDUCTASE (0.54×10^{-8} MOLES) WITH p-HMB

Sample	Solvent system	p-HMB required		-SH Groups/ mole of enzyme
		μl	Moles $\times 10^8$	
1.	Potassium phosphate buffer (pH 7.0)	60	2.30	4.1
2.	"	53	2.05	3.8
3.	"	52	2.00	3.7
				3.86 average
4.	Phosphate buffer (pH 7.0) + denaturant	56	2.15	4.0
5.	"	58	2.23	4.1
6.	"	58	2.23	4.1
7.	"	53	2.05	3.8
				4.0 average

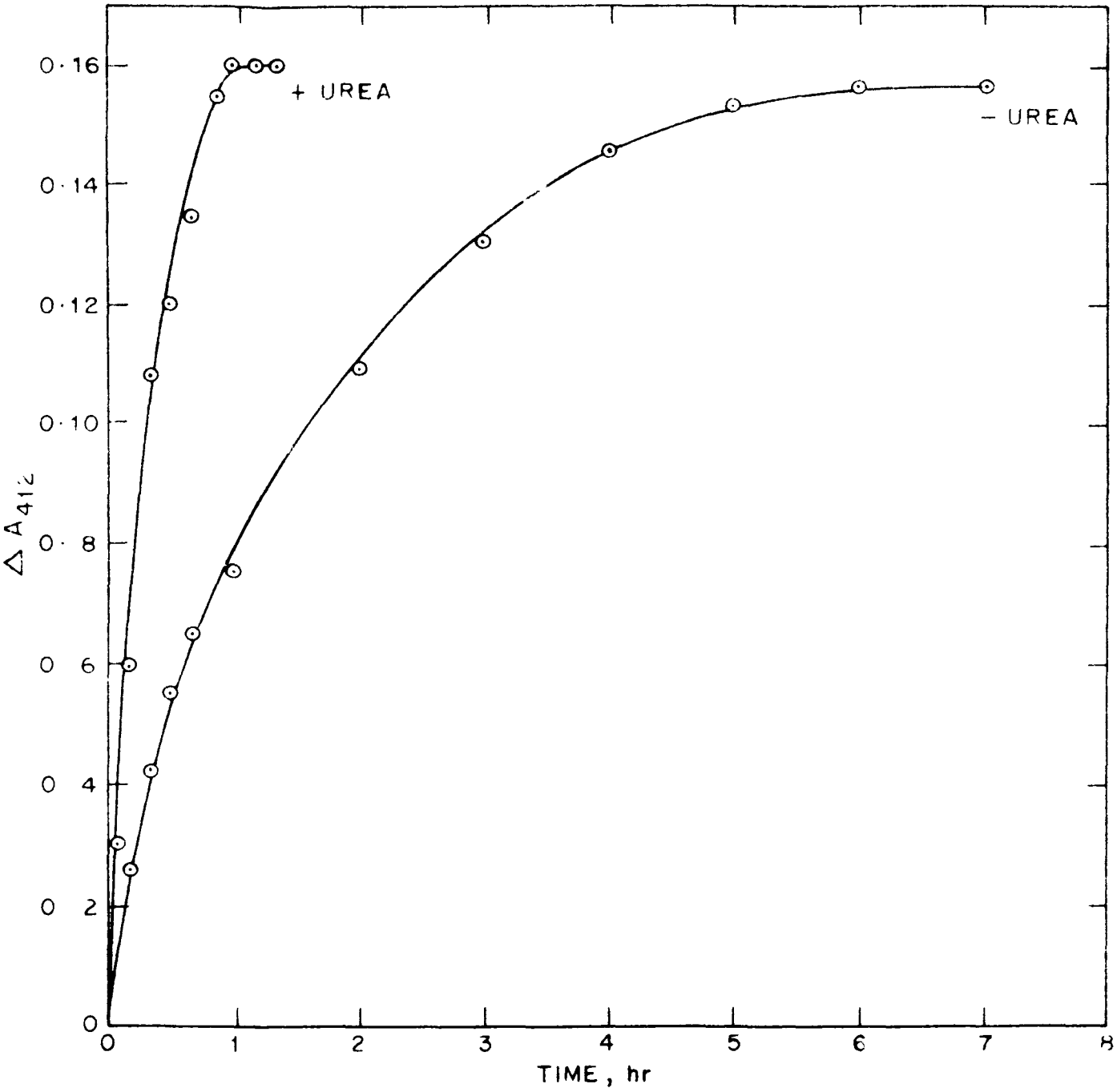


FIG. 16 RATE TITRATION OF A. FISCHERI NITRITE REDUCTASE WITH DTNB. The reaction of —SH groups was determined by absorption changes at 412 nm. Samples containing 0·26 mg protein in 1·0ml of 0·05 M potassium phosphate buffer (pH 7·5) containing 8 M urea (or no urea) DTNB (20μl, 10 mM) was added to the test samples and blanks and absorbancies recorded at 412 nm.

TABLE 15

HALF-CYSTINE AND FREE THIOL GROUPS IN *A. FISCHERI* NITRITE REDUCTASE

The number of experiments are reported in parentheses

Method	Residues/mole enzyme		
	Native enzyme[a] cysteine	Denatured enzyme[b]	
		Cysteine	Cysteine + half-cystine
I. With DTNB	3.6(3)	3.85(4)	5.9(2)[c]
II. With p-HMB	3.85(3)	3.98(4)	-
III. As cysteic acid	-	-	10.0

[a]Titrations of thiol content of nitrite reductase with p-HMB and DTNB were carried out (see text) in 50 mM potassium phosphate buffer, pH 7.0 and 7.5 respectively.

[b]Prior to titration with p-HMB and DTNB enzyme samples were preincubated for about 1 hr at room temperature in the buffer containing 8M urea or 8M urea plus 1% SDS.

[c]Estimated with DTNB after borohydride reduction in presence of 8M urea according to Cavallini *et al.* (287).

Determination of total —SH groups and —S—S— linkages

(i) $NaBH_4$ reduction and DTNB titration

The results of total half-cystine (— SH groups and —S—S— linkages) determinations are included in Table 15.

The reduction of the enzyme (1 mg) with $NaBH_4$ in urea followed by DTNB titration gave a value of 6 —SH groups per mole of enzyme. Assuming that the enzyme contains 4 free sulfhydryls as revealed by DTNB and p-HMB titrations, this would indicate the presence of one —S—S— linkage per mole of nitrite reductase.

(ii) Performic acid oxidation and cysteic acid determination

As shown in Table 15, analysis of performic acid-oxidized sample revealed 10 cysteic acid residues per mole of enzyme. The heme moiety is removed from heme protein by oxidation with performic acid (289). The value of 10 moles of cysteic acid per mole of enzyme on performic acid-oxidized protein also indicates the presence of one —S—S— linkage assuming that 4 —SH groups are involved in thioether linkages for binding the two heme moieties present in the enzyme molecule (170), each of the hemes being bound by two thioether linkages.

Inhibition of nitrite reductase activity by sulfhydryl reagents

The inhibitory effect of reagents which can react with sulfhydryl groups is shown in Table 16. Titration of all the 4 free -SH groups detected in the enzyme molecule with slight excess of p-HMB (4-5 moles/mole enzyme) or DTNB (50 moles/mole

TABLE 16

INHIBITION OF A. FISCHERI NITRITE REDUCTASE BY -SH REAGENTS AND ITS REVERSAL BY SULFHYDRYL COMPOUNDS AND DIALYSIS

Inhibitor	Inhibitor concentration (mole/mole enzyme)	Percent inhibition (BVH system)[a]
5,5'-Dithiobis(2-nitro benzoic acid)[b]	50	0
p-Hydroxymercuribenzoic acid[b]	5.5	0
p-Hydroxymercuribenzoic acid(170)	1650	35
p-Hydroxymercuribenzoic acid (170)	3300	95
p-Hydroxymercuribenzoic acid + cysteine or GSH (10 μmoles)[c] (170)	3300	2.5
p-Chloromercuribenzene sulfonic acid (170)	1350	94
p-Chloromercuribenzene sulfonic acid + cysteine or GSH (10 μmoles)[c] (170)	1350	4-5
p-Chloromercuribenzene sulfonic acid -dialyzed[d]	-	20

[a]Standard conditions of assay with reduced benzyl viologen (BVH) as electron donor were used (170).

[b]The enzyme was treated with DTNB (50 moles/mole enzyme, 4-5 hr) and p-HMB (5.5 moles/mole enzyme, 1 hr), until there was no further increase in absorption at 412 nm and 250 nm, respectively. Suitable aliquots were taken for testing the activity.

[c]The reversal of inhibition was achieved by adding the sulfhydryl compound and incubating for another 5 min before testing the activity.

[d]The enzyme (0.25 nmole) was preincubated in 1 ml 50 mM potassium phosphate buffer, pH 7.5, with the inhibitor (1.6 μmoles) for 10 min at 30°C. The enzyme was dialyzed overnight at 4°C against 50 mM potassium phosphate buffer, pH 6.8, with three changes of buffer before testing the activity.

enzyme) was found to have no effect on the nitrite reductase activity with reduced benzyl viologen as electron donor. However, in the presence of large molar excess (375- to 825-fold/—SH group) of the mercurial reagents, p-CMS and p-HMB, the enzyme activity was completely inhibited; this inhibition was completely reversed by subsequent incubation with —SH containing compounds, cysteine and reduced glutathione (170). The inhibition of nitrite reductase activity by sulfhydryl reagents was also reversed (70-80%) on removal of these reagents by dialysis. These results suggest that the inhibition by sulfhydryl reagents does not seem to be related to mercaptide formation but involves other interactions. It further suggests that no permanent derangement in the enzyme molecule is induced by the sulfhydryl reagents. Similar observations have been made in the case of other enzymes (323-325).

Partial specific volume

The partial specific volume ($\bar{v}$) of a protein can be calculated according to the method of Cohn and Edsall (299) from the weight percentages of the amino acid residues and their respective specific volumes. From the amino acid composition and the apparent specific volumes [$\bar{v}$ taken from Cohn and Edsall, (299)] a partial specific volume of 0.73 ml/g was calculated for A. fischeri nitrite reductase (Table 17). This is in good agreement with $\bar{v}$ values of 0.72 (168) and 0.73 ml/g (111) determined experimentally for P. aeruginosa nitrite reductase. The amounts of asparagine and glutamine used in these calculations were obtained by distributing the amide groups according to the

TABLE 17

PARTIAL SPECIFIC VOLUME OF A. FISCHERI NITRITE REDUCTASE

The 62 amide groups have been distributed according to the ratio found for glutamic acid to aspartic acid. Values for $\bar{v}$ for the amino acid residues were taken from Cohn and Edsall(299)

Amino acid residues	No. of residues/ mole of enzyme	Amino acid residue per 100 g of protein (weight percent)	$\bar{v}$	$\bar{v}$ x weight percent
Tryptophan	8	1.863	.74	1.379
1/2 Cystine	10	2.113	.61	1.289
Methionine	17	2.789	.75	2.092
Arginine	18	3.516	.70	2.461
Histidine	22	3.775	.67	2.529
Proline	24	2.914	.76	2.215
Tyrosine	26	5.306	.71	3.767
Phenylalanine	34	6.259	.77	4.819
Valine	41	5.086	.86	4.374
Glycine	42	3.000	.64	1.920
Serine	42	4.575	.63	2.882
Isoleucine	44	6.228	.90	5.605
Threonine	44	5.563	.70	3.894
Leucine	48	6.795	.90	6.115
Lysine	50	8.016	.82	6.573
Alanine	52	4.623	.74	3.421
Aspartic acid	54	7.772	.60	4.663
Asparagine	31	4.423	.62	2.742
Glutamic acid	55	8.879	.66	5.860
Glutamine	31	4.966	.67	3.327
		98.460		71.927

Partial specific volume of the enzyme was calculated as follows (ref. Chapter II)

$$\bar{\bar{v}} = \frac{71.927}{98.460} = 0.73 \text{ ml/g}$$

ratio found for aspartic acid to glutamic acid.

Theoretical titration curve and isoionic point:

The number of ionizable acidic and basic groups of A. fischeri nitrite reductase with their pK values are given in Table 18. Assuming independent ionization of the charged groups and neglecting electrostatic interactions and any end groups, a theoretical titration curve (Fig. 17) for A. fischeri nitrite reductase was constructed from the data of Table 18. For this hypothetical case, an isoionic point of 5.1 was calculated. This value is compatible with slight predominance of acidic over basic groups in the enzyme and may be compared with the determined isoelectric point of 4.5 using disc gel electrophoreis (170).

Degree of hydrophobicity

The degree of hydrophobicity of A. fischeri nitrite reductase in terms of average hydrophobicity, $H\emptyset_{av}$ (297), polarity index, p (295), and the frequency of non-polar side chains, NPS (296) was computed from the amino acid composition as shown in Table 19. Similar calculations were made for nitrite reductases from P. aeruginosa and C. fusca. The results are summarized in Table 20. The degree of hydrophobicities of the three enzymes as measured by the three parameters is strikingly similar. The $H\emptyset_{av}$ fall in the limited range between 1000 and 1100 cal/residue for globular proteins (297) and the same applies to Fisher's polarity index p (296) and Waugh's NPS (296). These results when interpreted according to Bigelow (297),

TABLE 18

IONIZABLE GROUPS OF *ACHROMOBACTER FISCHERI* NITRITE REDUCTASE

Ionizable group	Total number in nitrite reductase	pK[a] assumed
γ and δ Carboxyls	109[b]	4.5
Imidazolium	22	6.5
ε Ammonium	50	10.0
Phenolic hydroxyls	26	10.0
Sulfhydryls	4	10.0
Guanidinium	18	12.5

[a] pK values for the individual groups are taken from Mahowald, Noltmann and Kuby (300).

[b] Calculated as follows: 171 Carboxyls - 62 amide groups.

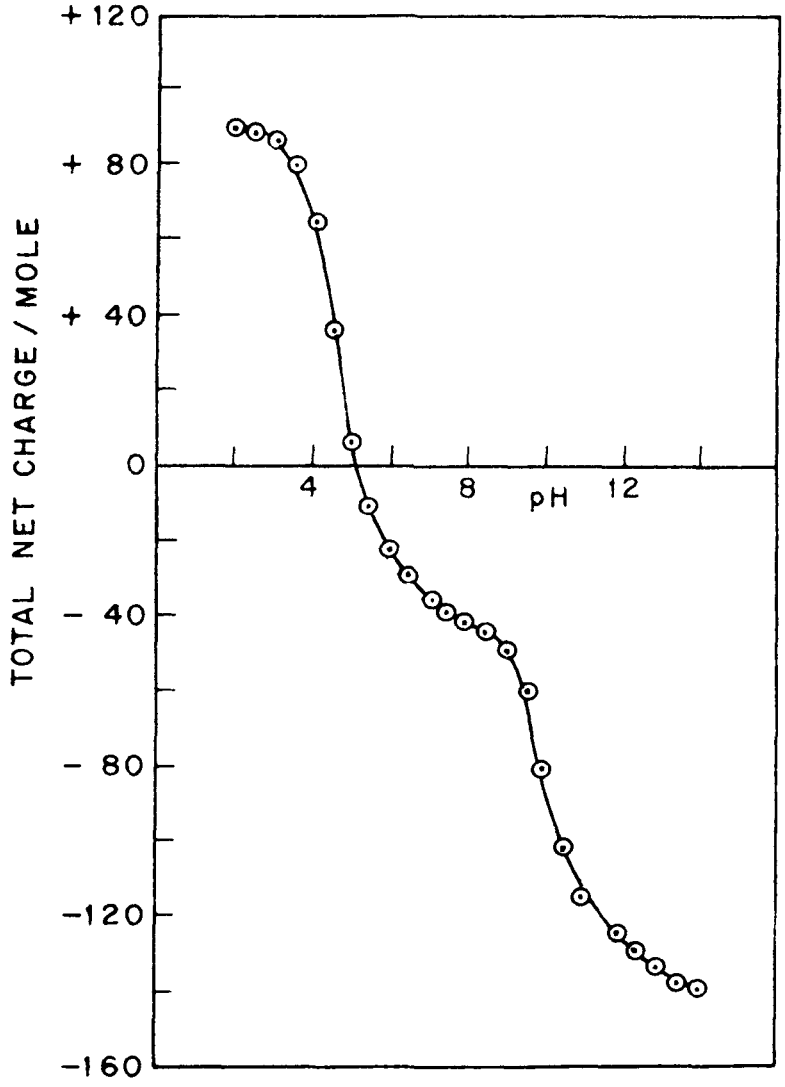


FIG. 17 THEORETICAL TITRATION CURVE FOR A. FISCHERI NITRITE REDUCTASE.

Calculations were based on the ionizable groups present in the enzyme (Table 18) pk values for the individual groups are taken from Mahowald, Noltmann, and Kuby (300).

TABLE 19

CALCULATION OF NPS, p AND $H\emptyset_{av}$ FOR *A. FISCHERI* NITRITE REDUCTASE

Amino acid	Number of residues	Volume ($Å^3$)	HØ(cal)
Non-polar			
Tryptophan	8	1083.2	24000
Isoleucine	44	4488.0	129800
Phenylalanine	34	3872.6	90100
Proline	24	1786.4	62400
Leucine	48	4896.0	115200
Valine	41	34891	69700
Methionine	17	1660.9	22100
Alanine	52	2735.2	39000
Glycine	42	1524.6	
1/2-Cystine	10	683.0	10000
		26198.2	
Polar			
Tyrosine	26	2021.2	74100
Lysine	50	5255.0	75000
Arginine	18	1963.8	13500
Threonine	44	3132.8	19800
Serine	42	2305.8	
Histidine	22	2021.8	
Aspartic acid	54	3693.6	
Glutamic acid	55	4658.5	
Amide	62	248.0	
		26300.5	
Total	693		744700

Non-polar and polar residues are separated according to Fisher's definition (295).
NPS is calculated according to Waugh's definition (296) by counting the trp, ile, tyr, phe, pro, leu and val residues and expressing the sum as a fraction of the total number of residues:
NPS = 225/693 = 0.32
p is the ratio of polar volume to non-plar volume =26300.5/26198=1.0
$H\emptyset_{av}$ is the total hydrophobicity divided by the number of residues (297) = 744700/693 = 1075 cal/res.

TABLE 20

DEGREE OF HYDROPHOBICITIES FOR A. FISCHERI, P. AERUGINOSA AND C. FUSCA NITRITE REDUCTASES AS MEASURED BY THE THREE PARAMETERS

Proteins	$H\emptyset_{ave}$ (297) cal/res	p (295)	NPS (296)
Nitrite reductase			
A. fischeri	1075	1.00	0.32
P. aeruginosa	1107	0.91	0.35
Chlorella fusca	1079	0.96	0.32

The numbers in parentheses are the appropriate references

Fisher (295), and Waugh (296) suggest globular nature of the three nitrite reductases.

DISCUSSION

The amino acid composition of A. fischeri nitrite reductase, calculated by Markland and Damus' method and those from P. aeruginosa (167) and C. fusca (135) (whose amino acid compositions have been reported) are shown in Table 21. Although there are differences in the amino acid compositions of the three nitrite reductases, the overall amino acid composition is quite similar when comparison is made on weight per cent basis. In this respect the respective contents of threonine, isoleucine, lysine, and aspartic acid are particularly noteworthy, the variation being ± 5%. The percentage composition of methionine, histidine, valine, serine, leucine, and glutamic acid agrees within ± 10%, while those of alanine, tyrosine and glycine agrees within ± 15%. The number of hydrophobic amino acid residues in all the three enzymes is comparable. The tryptophan content of Pseudomonas enzyme is considerably higher as compared to Achromobacter and Chlorella enzymes. A striking feature in the amino acid composition of Chlorella nitrite reductase is its high alanine and glycine contents as compared to the other two nitrite reductases. The number of proline residues in Achromobacter enzyme is just half of that in Chlorella and Pseudomonas enzymes.

TABLE 21

AMINO ACID COMPOSITION OF NITRITE REDUCTASES FROM *A. FISCHERI*, *P. AERUGINOSA* AND *C. FUSCA*

Amino Acid Res.	*A. fischeri* (80,000)	*P. aeruginosa*[a] (67,325)	Chlorella[b] (63,000)
Trp	8	15(18)	4(5)
H. Cys	10	2(2)	10(13)
Met	17	12(14)	13(17)
Arg	18	24(29)	29(37)
His	22	15(18)	13(17)
Pro	24	40(48)	36(48)
Tyr	26	21(25)	14(18)
Phe	34	15(18)	23(30)
Val	42	45(53)	39(50)
Gly	42	47(56)	56(71)
Ser	42	42(50)	27(34)
Ile	44	33(49)	31(39)
Thr	44	36(43)	34(43)
Leu	48	40(48)	49(62)
Lys	50	40(48)	38(48)
Ala	52	46(55)	60(76)
Asp	86	67(80)	61(77)
Glu	86	57(68)	63(80)

Number of amino acid residues in parenthesis have been calculated per 80,000 mol. wt.

[a]Data of Nagata *et al.*(167)

[b]Data of ~~M.G.~~ Zumft (135).

All the three nitrite reductases are rich in dicarboxylic acids. The difference in the content between basic (lysine + arginine + histidine = 90 residues) and acidic (aspartic + glutamic - amides = 109 residues) amino acid residues in A. fischeri nitrite reductase showed an excess of acidic residues which is in accord with its acidic isoelectric point of about pH 4.5 (170). The isoelectric pH of Pseudomonas enzyme has not been reported. In the case of Chlorella enzyme the aspartic and glutamic acid contents are also in excess and consistant with its isoelectric pH of about 5.0 (135).

The number of half-cystine residues of nitrite reductases from A. fischeri, C. fusca and P. aeruginosa are 10,10 and 2 respectively. Out of a total of 10 half-cystine residues present in A. fischeri enzyme, 4 residues are present as free sulfhydryl groups, two are accounted as one disulfide bridge and the remaining 4 cysteine residues may be involved in binding the two heme c moieties of the protein. The free thiol groups are not essential for enzyme activity as the enzyme is completely active when the four —SH groups are titrated with p-HMB or DTNB. The depressing effect caused by relatively high concentrations of p-HMB and p-CMS on nitrite reductase activity reported earlier (170) appears to be due to nonspecific action of the inhibitors and does not involve interaction with —SH groups of the enzyme. Similar effect has been observed in the case of other enzymes (323-325). Neither free -SH nor —S—S— groups

were detected in *Pseudomonas* nitrite reductase. The two half-cystine residues present in this enzyme have been implicated in the binding of heme *c* moiety to the protein. The half-cystine content of *Chlorella* nitrite reductase was determined with performic acid-oxidised protein. No attempt was made to estimate the free sulfhydryl groups or —S—S— linkages. The fact that the *Chlorella* nitrite reductase activity was completely blocked by 0.1 mM p-HMB has been interpreted to indicate the presence of free sulfhydryl groups which have been postulated to participate in the electron transfer and in binding the two iron atoms to the apoenzyme (135).

The value of hydrophobicities in terms of $H\emptyset_{av}$, p, and NPS for the three nitrite reductases are quite similar and fall within their respective ranges for globular proteins suggesting the globular nature of the enzymes.

CHAPTER 6

REVERSIBLE INACTIVATION

SUMMARY

A. fischeri nitrite reductase is markedly inactivated at acid pH and on treatment with urea, Gu.HCl, and SDS at relatively low concentrations. Gu.HCl is more effective on a molar basis than urea. Phosphate, nitrite, and hydroxylamine markedly protect the enzyme from inactivation by 2 M urea. The optimal concentrations of phosphate and the substrates for the protection of the enzyme against urea-inactivation are 0.5 M, 5 mM, and 20 mM respectively.

The kinetic analyses show that the inactivation involves 2 moles of urea per mole of enzyme and is of noncompetitive type with a Ki of 1.45-1.6 M. The ΔH_a for the inactivation of the enzyme in 2 M urea is 9.2 kcal compared to 60 kcal obtained in the absence of urea.

The inactivation of the enzyme by 2-4 M urea and 1-2 M Gu.HCl is reversible. As much as 90 to 95% of the initial activity is recovered when urea is removed by dialysis. Only 50% to 60% reversal is achieved with Gu.HCl-treated enzyme. Inactivation of the enzyme with SDS is, however, irreversible suggesting that the action of SDS is qualitatively different from that of urea and Gu.HCl.

The inactivation of the enzyme at acid pH is also reversed on neutralization. About 70 to 75% reversal is achieved under optimal conditions. The presence of bovine serum albumin at a concentration of 1.0 mg/ml in the reactivation mixture is

an absolute requirement for the reversal. The rate and extent of the reactivation depend upon the length of time the enzyme is exposed to acid pH.

The Km, and the ratio of nitrite reductase to hydroxylamine reductase activities of the renatured enzyme are same as that of the native enzyme.

INTRODUCTION

Denaturation studies are capable of yielding information about the native state of a protein in terms of its co-operativity, intrinsic stability and the nature of forces responsible for maintaining its tertiary structure.

A change in the native structure of a protein is referred to as 'denaturation'. This process is generally accompanied by a change in the optical properties, structure, and loss of biological activity.

Denaturation can be brought about by modification of the solvent (addition of organic solvents, salts, urea or Gu.HCl) or by a change in pH, by addition of detergents, heavy metal ions, complexing agents or by a temperature change. Denaturation can also be brought about by a chemical modification of the protein such as oxidative or reductive cleavage of disulfide groups, oxidation of thiol groups to disulfide or sulfonic acid groups, and substitution of functional groups.

Urea and Gu.HCl are the most commonly used protein denaturants. It is widely believed that these reagents act as protein denaturants by breaking the intramolecular hydrogen bonds (326). However, it has been shown that the denaturing effectiveness of different compounds does not follow the order expected for hydrogen bond formation between the denaturing agent and the protein (327). Urea increases the solubility of non-polar compounds in aqueous solution and it has been

suggested that this hydrophobic effect contributes to the denaturation of proteins by urea (328,329). The denaturing ability of urea, however, cannot be attributed solely to its hydrophobic effects (330,331). It has been shown that urea decreases the activity coefficients of peptide and amide groups (328) and that this non-hydrophobic effect makes a major contribution to the denaturing action of compounds of urea-guanidinium class (332).

Of the two denaturants, Gu.HCl has been conclusively shown to be more effective. Thus, the minimum concentration of urea required to completely denature bovine serum albumin (BSA) was found to be 8.5 M, whereas the same effect could be produced by 5.3 M Gu.HCl (333). Similarly, the concentrations of urea and Gu.HCl to cause complete inactivation of isocitrate lyase are reported to be 4.0, and 1.6 M respectively, indicating that Gu.HCl is a much more stronger denaturant (334).

Detergents do not compete for peptide bonds but weaken the hydrophobic bonds resulting in denaturation of proteins (330). The binding of ionic detergents may, however, disrupt hydrogen bonds indirectly as a result of intramolecular electrostatic repulsion (335). Detergents exert high denaturing action at relatively low concentrations as compared to urea and Gu.HCl. For instance, the intrinsic viscosity (η) of BSA is increased from 4.3 to 22 by 8 M urea and to 25 by 0.17 M SDS (336).

Denaturation at extreme pH is believed to be the result of the mutual repulsion between the charges proteins carry under such conditions.

The question of what factors control protein folding and conformation is of prime importance in biochemistry since a specific tertiary structure is required for an enzyme to be active. The phenomenon of reversible enzyme denaturation provides a powerful tool for studying protein folding, the final step in protein biosynthesis.

Sela, White, and Anfinsen (337) were the first to demonstrate that reduced and denatured ribonuclease could be refolded with full biological activity. Since these pioneering studies, complete disruption and reversal have been accomplished for a number of proteins including enzymes. Among the disulfide-containing proteins, the reduced chains of egg white lysozyme (338), taka-amylase (339), alkaline phosphatase (340), and β-galactosidase (341) have been shown to regain their native conformations and biological activities on removal of the denaturant and air oxidation. Similar results have been obtained with proteins which lack these internal linkages such as aldolase (342), enolase (343), glucose-6-phosphate dehydrogenase (345), acetoacetate decarboxylase (344), and bacterial luciferase (346).

These successful renaturation studies lend support to the theory (347) that the higher orders of structure in a native protein are determined thermodynamically by the amino

acid sequence of its polypeptide chain(s).

Renaturation of enzymes has been studied in great detail. The presence of substrates, co-factors, salts, and sulfhydryl reagents in the reactivation mixture as well as other conditions (temperature, pH, protein concentration etc) have been shown to influence the renaturation process (348-352).

Several enzymes possessing the same amino acid sequence have been found to refold into different conformational forms. Multiple forms of enzymes have been reported such as lactate (353), malate (354), and glyceraldehyde-3-phosphate (355) dehydrogenases. Similarly, several enzymes such as lactate dehydrogenase (356), ribonuclease (357), and creatine kinase (358) refolded in vitro following partial denaturation, have been reported to regain biological activity but possessed a conformation distinct from that of the native enzyme. These observations indicate that the enzymes were able to assume more than one active conformation supporting the idea that factors other than the primary structure play a significant role in the folding of proteins.

Studies on the denaturation and renaturation of A. fischeri nitrite reductase were undertaken. The results are presented in this chapter. The enzyme is inactivated at acid pH, and on treatment with protein denaturants such as urea, Gu.HCl, and SDS. The inactivation of the enzyme by urea has been studied in relation to changes in concentrations of the denaturant, enzyme, phosphate, substrates, sulfhydryl reagents as well as temperature

and pH. The inactivation at acid pH, and by urea and Gu.HCl is reversed on neutralization or by removal of the denaturants by dialysis. The properties of the urea-, and acid-denatured-renatured enzymes are determined and compared with those of the native enzyme.

METHODS

Inactivation of enzyme with urea, Gu. HCl, and SDS and its reversal

The effect of denaturants on nitrite reductase activity in prior-incubation system was studied as follows, unless stated otherwise. The enzyme samples were incubated for 10 min at 5°C in 0.1 M potassium phosphate buffer (pH 6.8) containing varying amounts of the denaturing agents. Aliquots were withdrawn and assayed for the residual activity. A control with the enzyme at the same dilution in the absence of the denaturing agent was always run. The residual activities were expressed as percent of the initial activity.

To examine the time course of the action of denaturants, the incubation was continued for a period of about 1-2 hr and inactivation followed by assaying aliquots withdrawn at different intervals. The controls were stable within this period of time; only slight inactivation was observed on prolonged incubation.

Inactivation in the assay system refers to incubation of the enzyme (0.2 to 2.0 μg) with urea in the assay mixture containing in 1.5 ml, 200 μmoles of potassium phosphate (pH 7.5) and 0.6 μmoles of sodium nitrite. After a preincubation of 2 to 3 min, the assay was started by tipping 0.5 ml of benzyl viologen (10 mg/ml) and 1.0 ml of freshly prepared dithionite (1 mg/ml). The assay was performed for 4 to 6 min at 30 to 32°C. The concentrations of urea indicated

in experiments refer to that present before benzyl viologen and dithionite additions which result in a two-fold dilution. It was observed that the denaturants at concentrations present in the assay mixture did not interfere with the colorimetric determination of nitrite.

Reactivation of the urea-, and Gu.HCl-treated enzyme was carried out by removing the denaturants by dialysis. The dialysis was performed against 0.05 M potassium phsophate buffer (pH 6.8) for 18 to 24 hr at 3-5°C.

Inactivation of enzyme at acid pH and its reversal

For studying acid inactivation of nitrite reductase, the enzyme (5 to 10 μg) was brought to the required pH by the addition of 10 volumes of 0.1 M acetate (pH 4.4 or 4.7). At suitable intervals, samples were withdrawn and residual activities determined.

For reactivation studies, the acid-treated enzyme was transferred into an equal volume of 0.2 M potassium phosphate buffer (pH 6.8) containing 0.1% BSA and the mixture incubated at 10-15°C. The regain of enzyme activity was followed by assaying 50-100 μl samples at suitable intervals.

RESULTS

The kinetics of denaturation was monitored by following the loss of enzyme activity. Similarly, renaturation of the denatured enzyme was followed by the regain of enzyme activity. The procedure for enzyme assay is described in Materials and Methods.

Effect of urea, Gu.HCl and SDS on enzyme activity

A. fischeri nitrite reductase is susceptible to inactivation by urea, Gu.HCl, and SDS. The effect of these denaturing agents on the activity of nitrite reductase was examined under conditions described in legends to Figs. 18a and 18b. It is apparent that all these denaturants caused marked inactivation of the enzyme at relatively low concentrations. Thus, the enzyme loses 50% of its initial activity in 2 M urea whereas the concentration of Gu. HCl required to produce the same effect was only 0.75 M indicating the latter to be a more potent denaturant. Treatment of nitrite reductase with urea and Gu.HCl at concentrations above 4.0 M and 2.0 M, respectively, resulted in instantaneous inactivation of the enzyme.

Since SDS tends to precipitate at low temperature, inactivation of the enzyme with this reagent was carried out at 15°C. The detergent had a pronounced effect on enzyme activity at very low concentrations. Thus, a concentration of only 0.01% (about 4.5×10^{-4} M) was required to produce 50% inactivation of the enzyme.

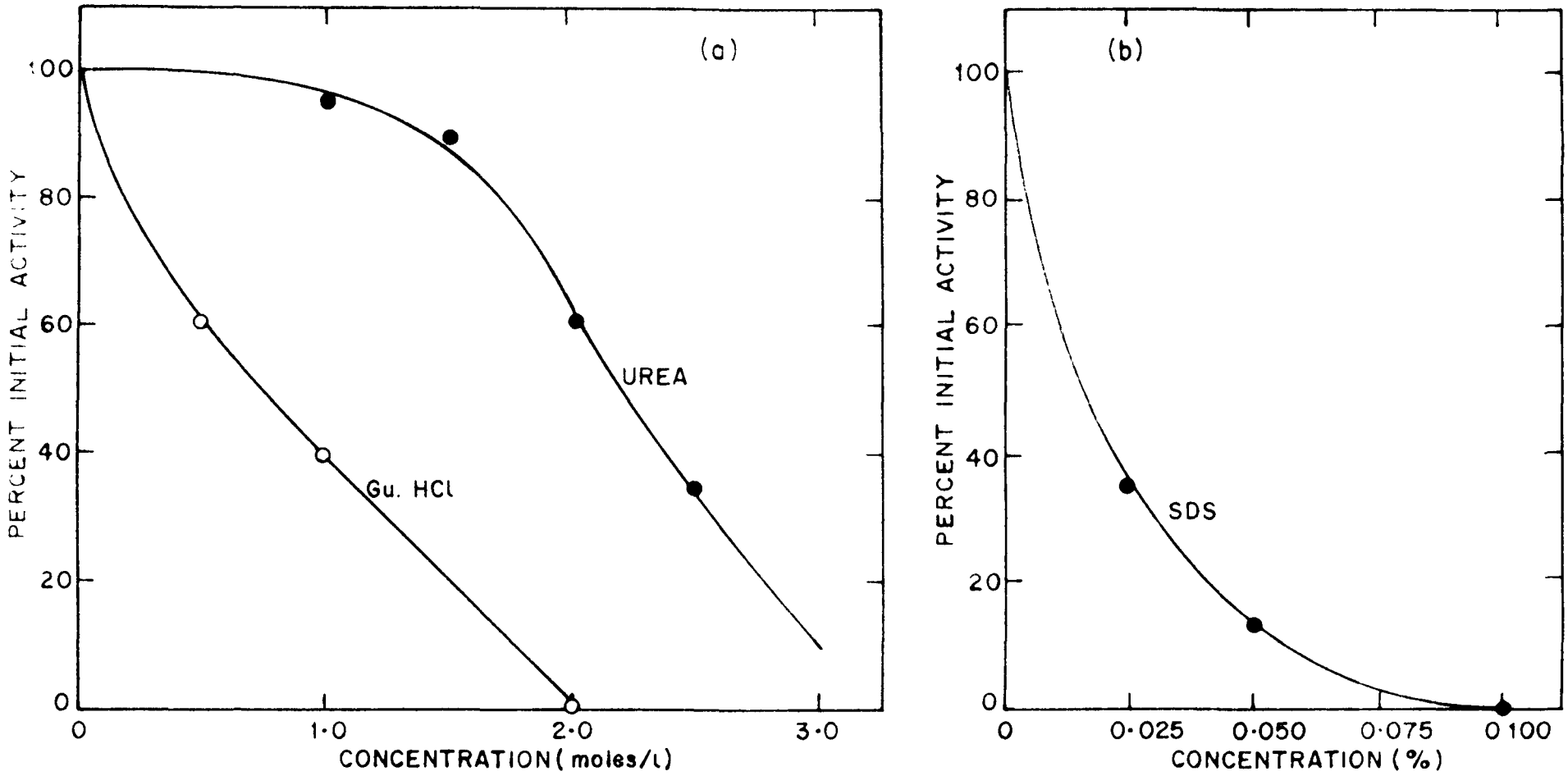


FIG. 18 INFLUENCE OF UREA, Gu.HCl, AND SDS ON THE ACTIVITY OF NITRITE REDUCTASE.

The enzyme (4 μg) in 0·8 ml of 0·1 phosphate buffer (pH 6·8) was incubated at 5 °C with urea and Gu HCl and at 15 °C with SDS at the concentrations of denaturants indicated. The activity was determind after 10 min incubation on suitable aliquots

The inactivation of the enzyme by direct addition of urea in the assay mixture (assay system) is shown in Fig. 19. About 50% inactivation was caused in 1.0 M urea as against 2.0 M required in the prior-incubation system. The greater susceptibility of the enzyme in the assay system is probably due to the higher temperature (30-32°C) used.

From Fig. 20(insert), it can be seen that inactivation by urea and Gu.HCl is linear with the logarithm of concentration. However, when inhibition data are formulated in a Dixon plot (359) as shown in Fig. 20, curves are obtained instead of straight lines. A deviation of this type may be associated with a reaction between the inhibitor and the substrate, which in the present case seems unlikely, or when more than one molecule of the reagent reacts with one molecule of the enzyme (360).

In order to determine the mode of inhibition by urea, the inhibition studies were carried out in the presence of varying concentrations of nitrite. When the data were analysed by the Lineweaver-Burk (361) (Fig. 21) and Laidler's (362) (Fig. 22) procedures, the inhibition by urea was found to be of noncompetitive type with respect to nitrite. The Ki for urea by the two methods was calculated to be 1.6 and 1.45 M respectively.

Analyses by the method of Johnson, Eyring and Williams (363) (Fig. 23) gave linear plots for points upto 1.2 M urea

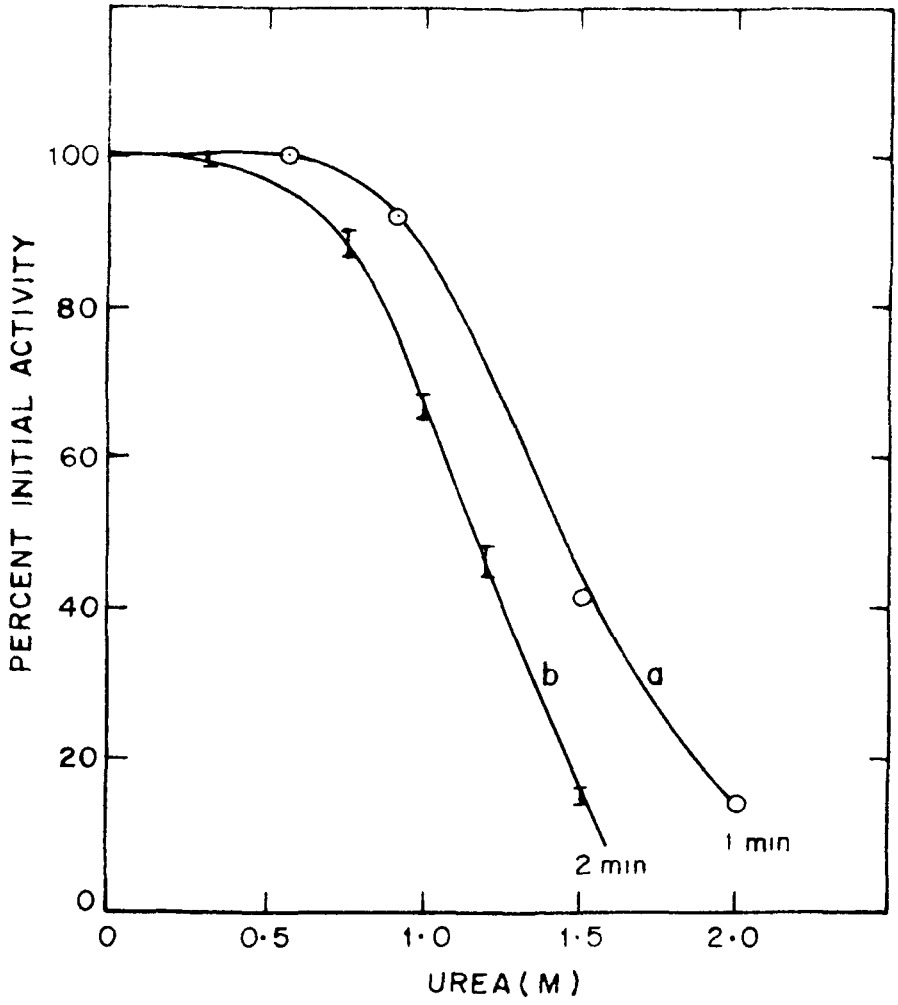


FIG 19 INFLUENCE OF UREA ON THE ACTIVITY OF NITRITE REDUCTASE IN THE ASSAY SYSTEM. The assay mixtures contained the indicated concentrations of urea. The activity of the enzyme was determined after 1 (a) and 2 min (b) of preincubation in the assay mixture containing the denaturant at 30–32 °C.

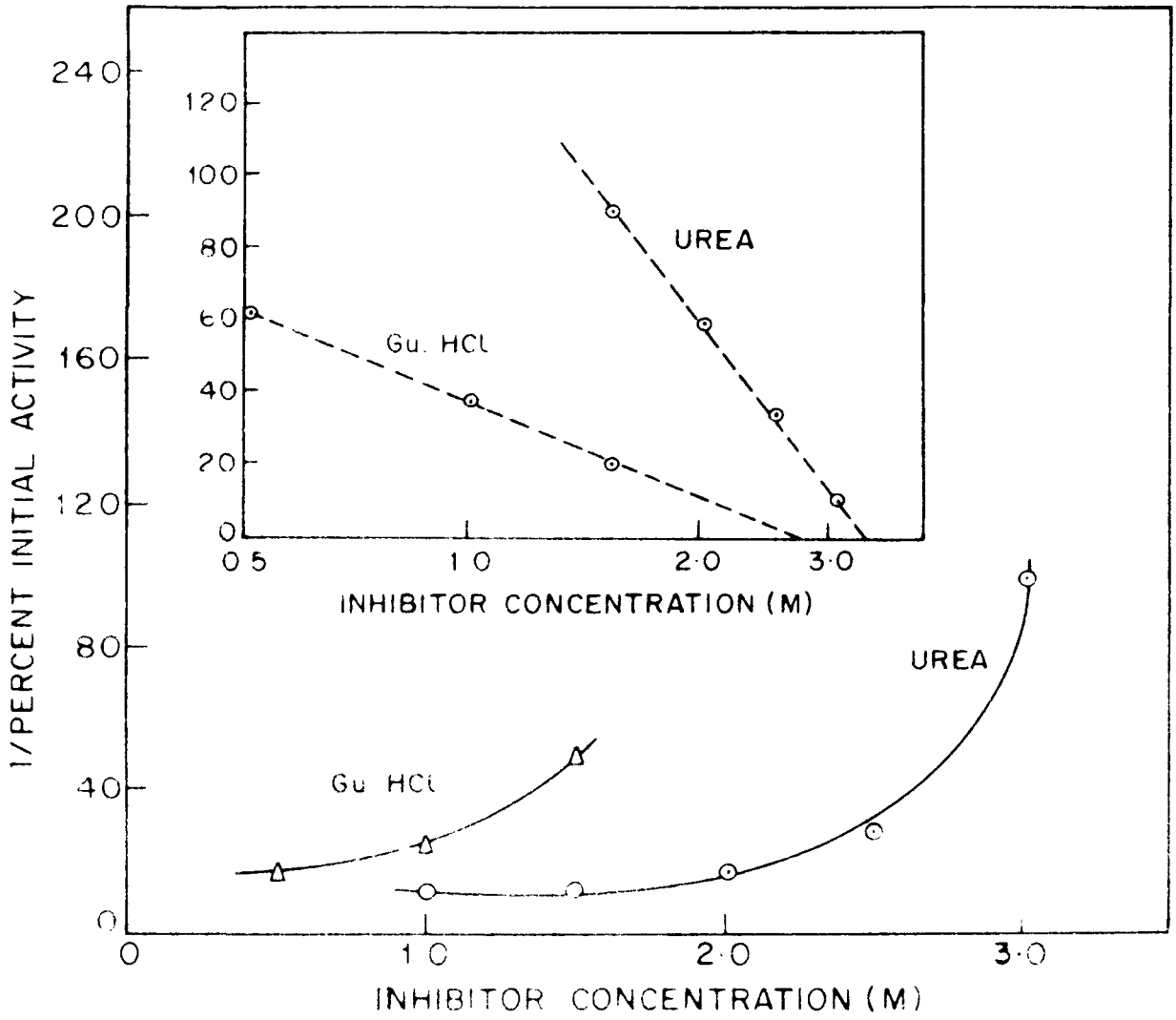


FIG 20 DIXON PLOTS OF INHIBITION OF NITRITE REDUCTASE ACTIVITY BY UREA & Gu.HCl IN THE PRIORINCUBATION SYSTEM. The insert shows the log concentration-dependence curves for urea and Gu. HCl.

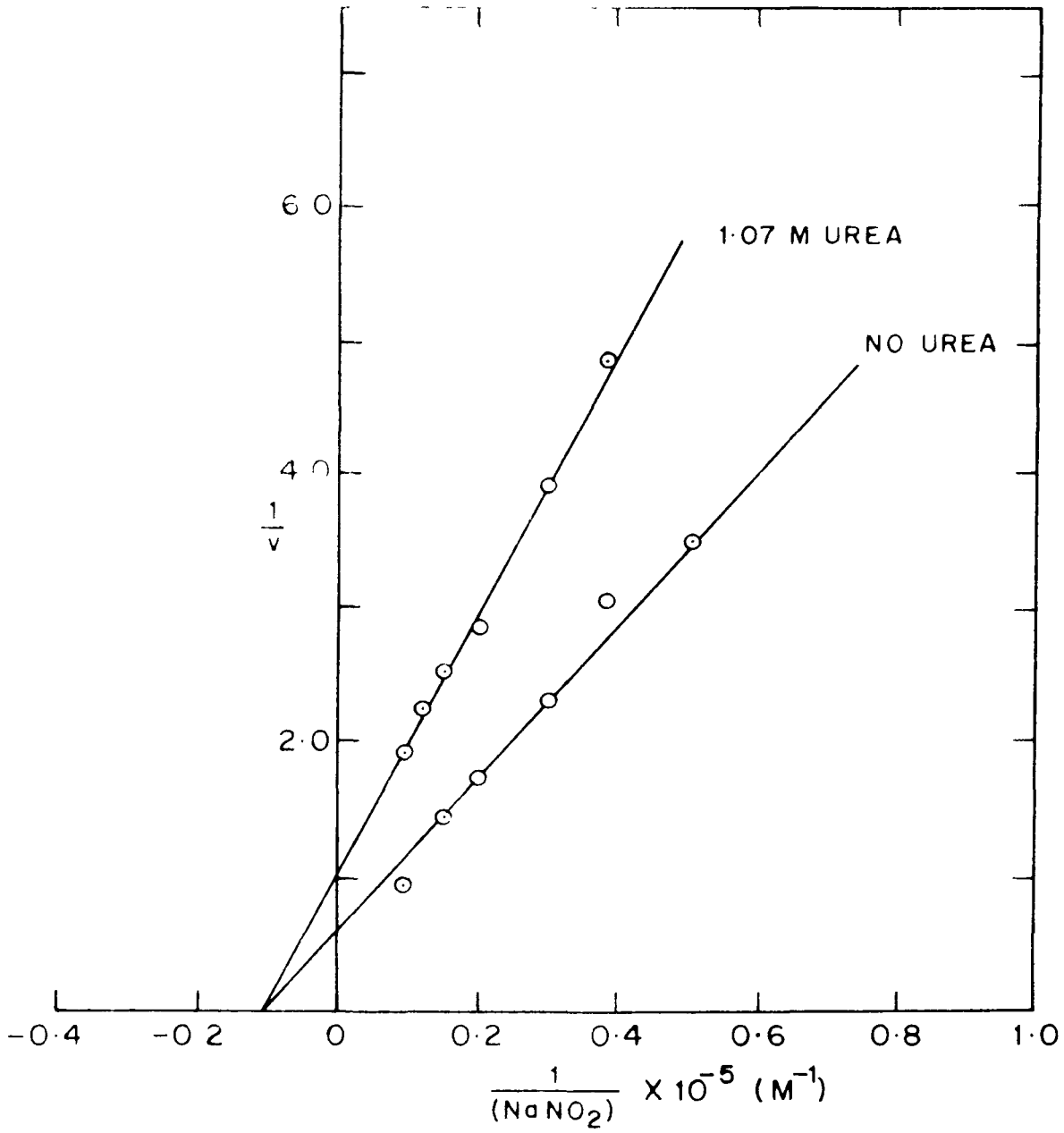


FIG 21 MODE OF INHIBITION BY UREA OF NITRITE REDUCTASE WITH RESPECT TO NITRITE.

Lineweaver–Burk plot for enzyme activity in the absence and presence of 1·07 M urea

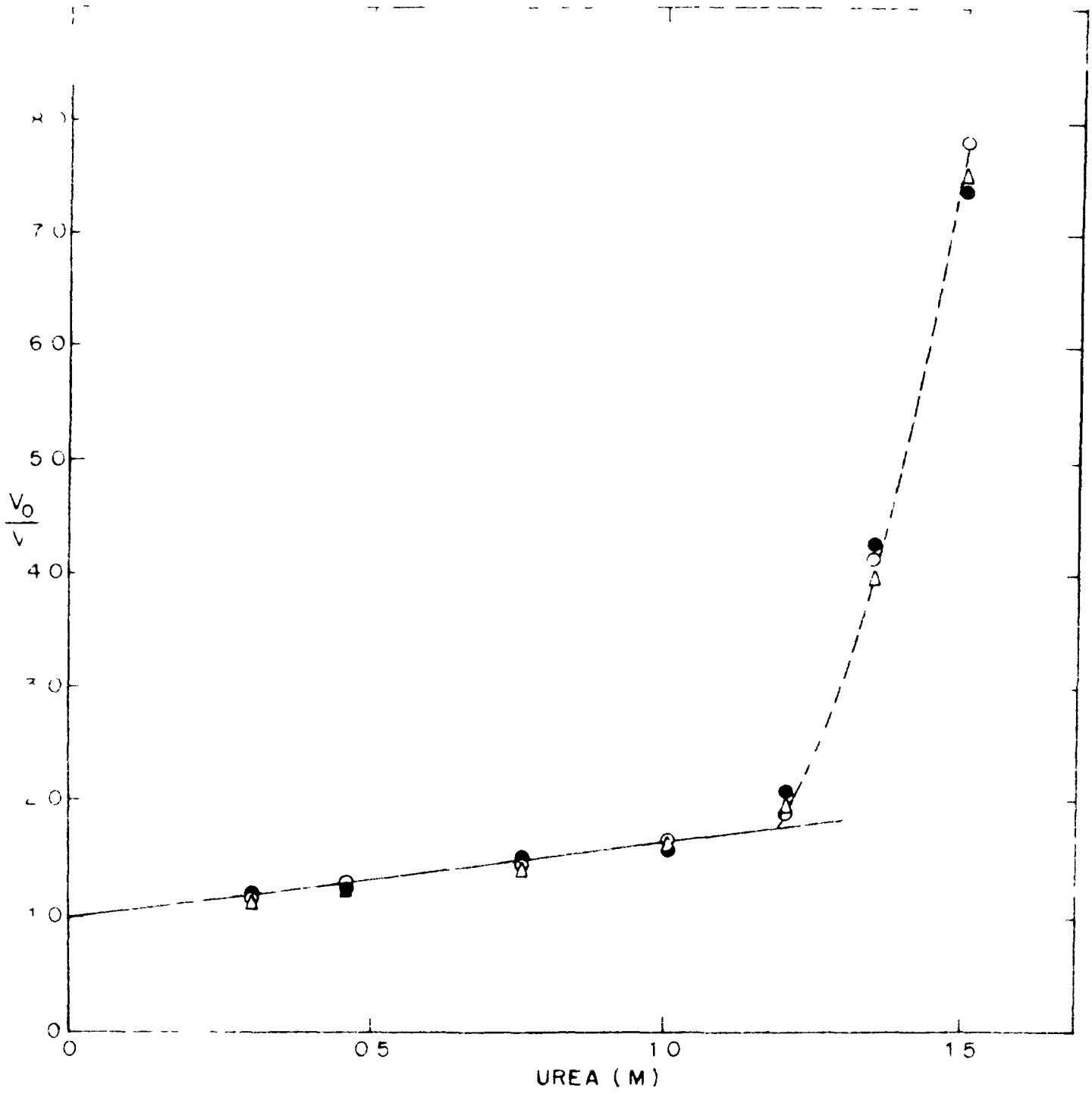


FIG 22 PLOT OF RELATIVE ACTIVITY, V_0/V AGAINST UREA CONCENTRATION IN THE PRESENCE OF DIFFERENT AMOUNTS OF SUBSTRATE [illegible], 0 4 (● , AND 1 0 mM (Δ). V & V_0 REFER TO THE NITIAL VELOCITY OF THE REACTION IN THE ABSENCE AND PRESENCE OF UREA RESPECTIVELY

with the slope ranging between 1.7 and 1.9, indicating that about 2 molecules of urea are involved in the observed inhibition. Beyond 1.2 M urea, sharp changes are again seen, with a large number (12-15) of urea molecules being apparently concerned.

The effect of enzyme concentration on the inhibition produced by urea in the assay system plotted according to the procedure of Ackermann and Potter (364) is shown in Fig. 24. Inhibition by urea appears to be irreversible with respect to enzyme.

Time dependence of inactivation of nitrite reductase with urea, Gu.HCl and SDS

The rates of inactivation of the nitrite reductase in urea, Gu.HCl, and SDS are shown in Fig. 25. The loss of enzyme activity was time-dependent and the rate of enzyme inactivation was a function of concentration of the denaturant. The process of inactivation was apparently first order as illustrated in Fig. 26. The rate constants derived from the data in Fig. 26 were 0.46 and 3.9 min^{-1} for 1 and 2 M urea, and 6.1 and 10.4 min^{-1} for 1.0 M Gu.HCl and 0.025% SDS respectively.

Further experiments were directed to study the loss of enzyme activity by urea in relation to changes in pH, temperature, and concentrations of the denaturant, enzyme, sulfhydryl reagents and the substrates. Inactivation with 2.0 M urea was moderate and likely to reflect conditions causing both increases and decreases in the stability of enzyme and was therefore chosen for subsequent experiments.

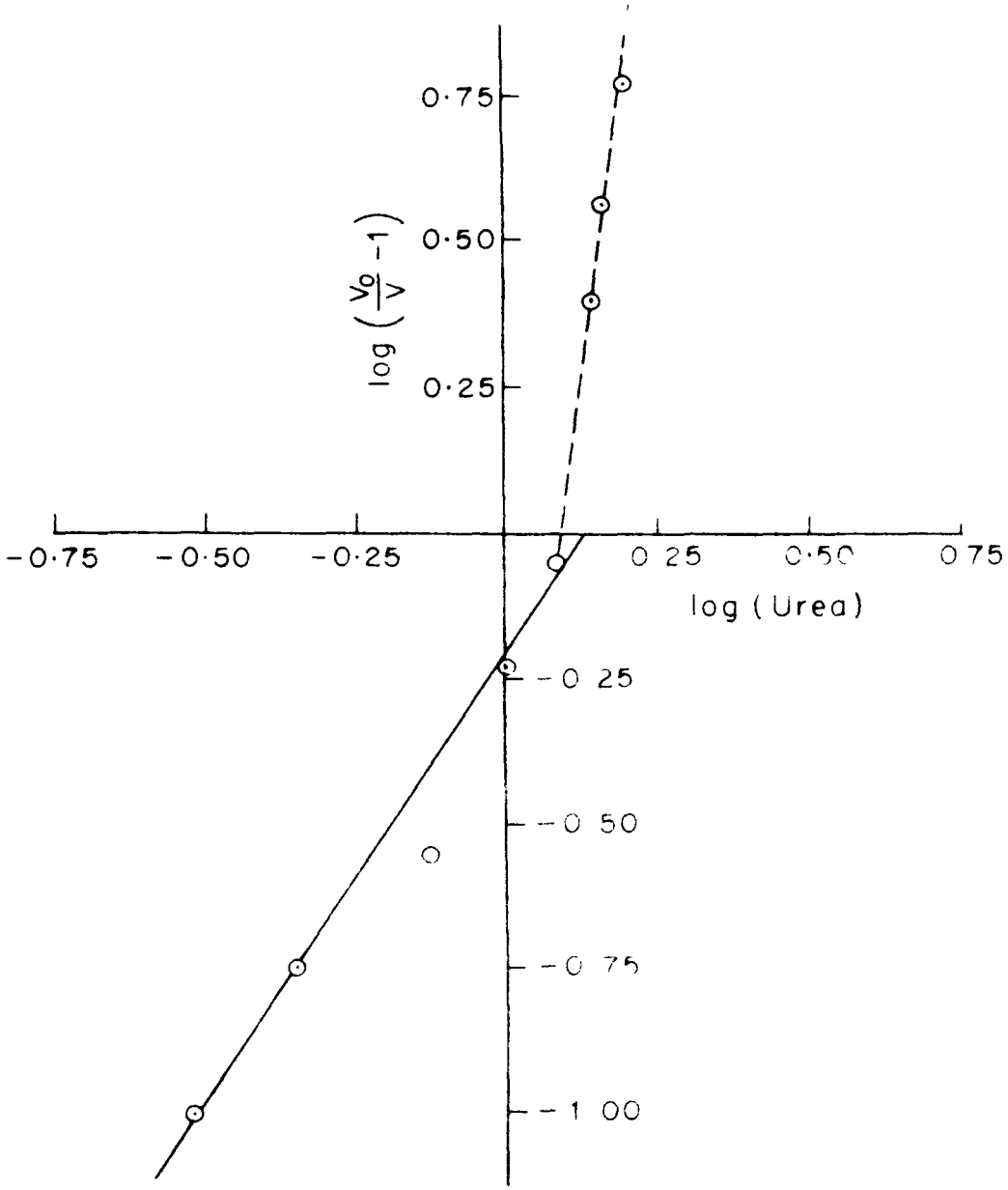


FIG. 23 JOHNSON-EYRING-WILLIAMS PLOT OF UREA INHIBITION
Data of Fig 22 have been replotted V_0 and V represent the initial velocity of the reaction in the absence and presence of urea Abscissa is the log of the urea activity (M) The treatment used assumes an equilibrium between active and urea-inactivated enzyme and negligible disturbance of this equilibrium by the reaction with substrate

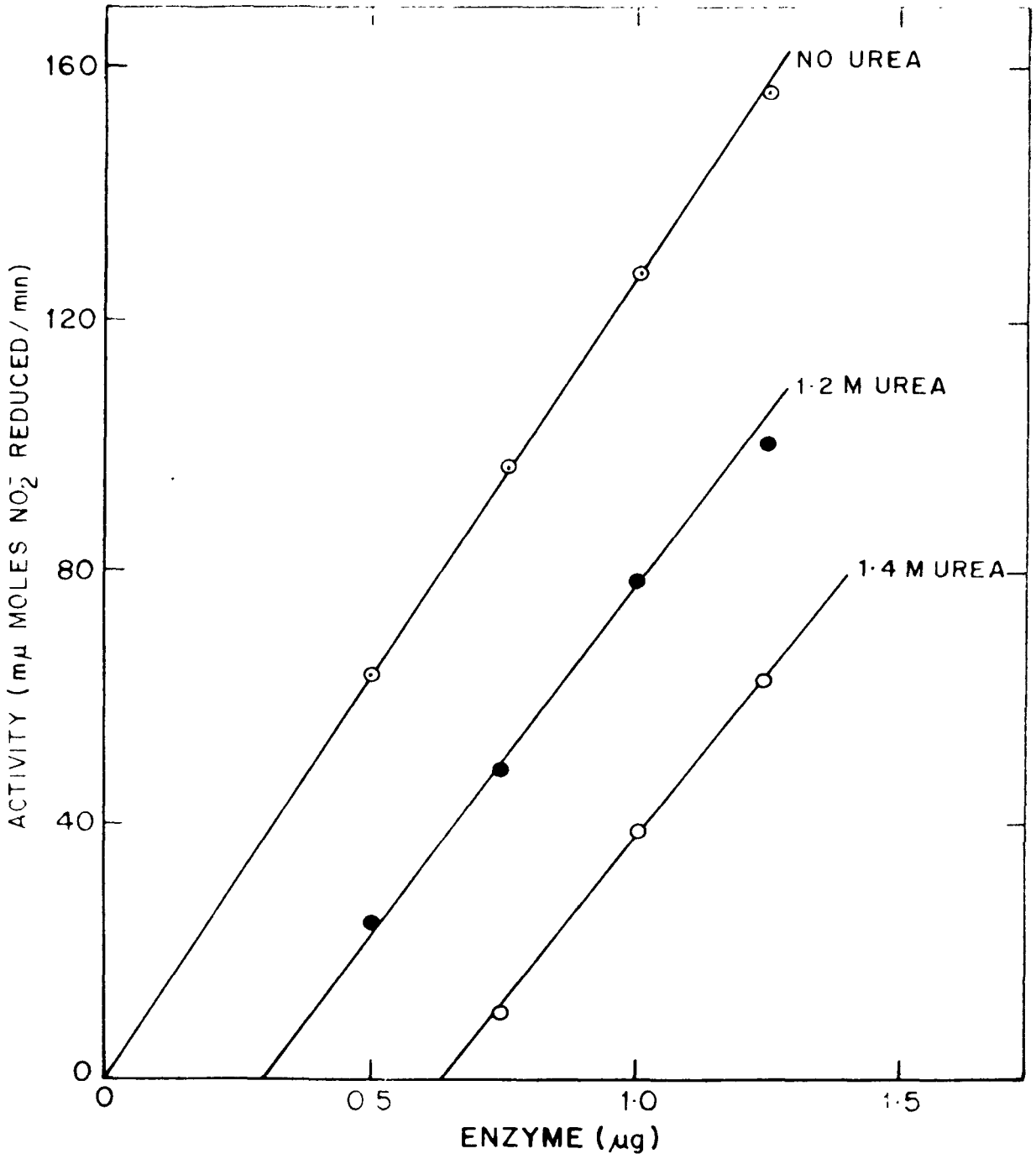


FIG. 24 ACKERMANN – POTTER PLOT OF INHIBITION BY UREA AT 33 °C IN THE ASSAY SYSTEM SHOWING IRREVERSIBILITY WITH RESPECT TO ENZYME.

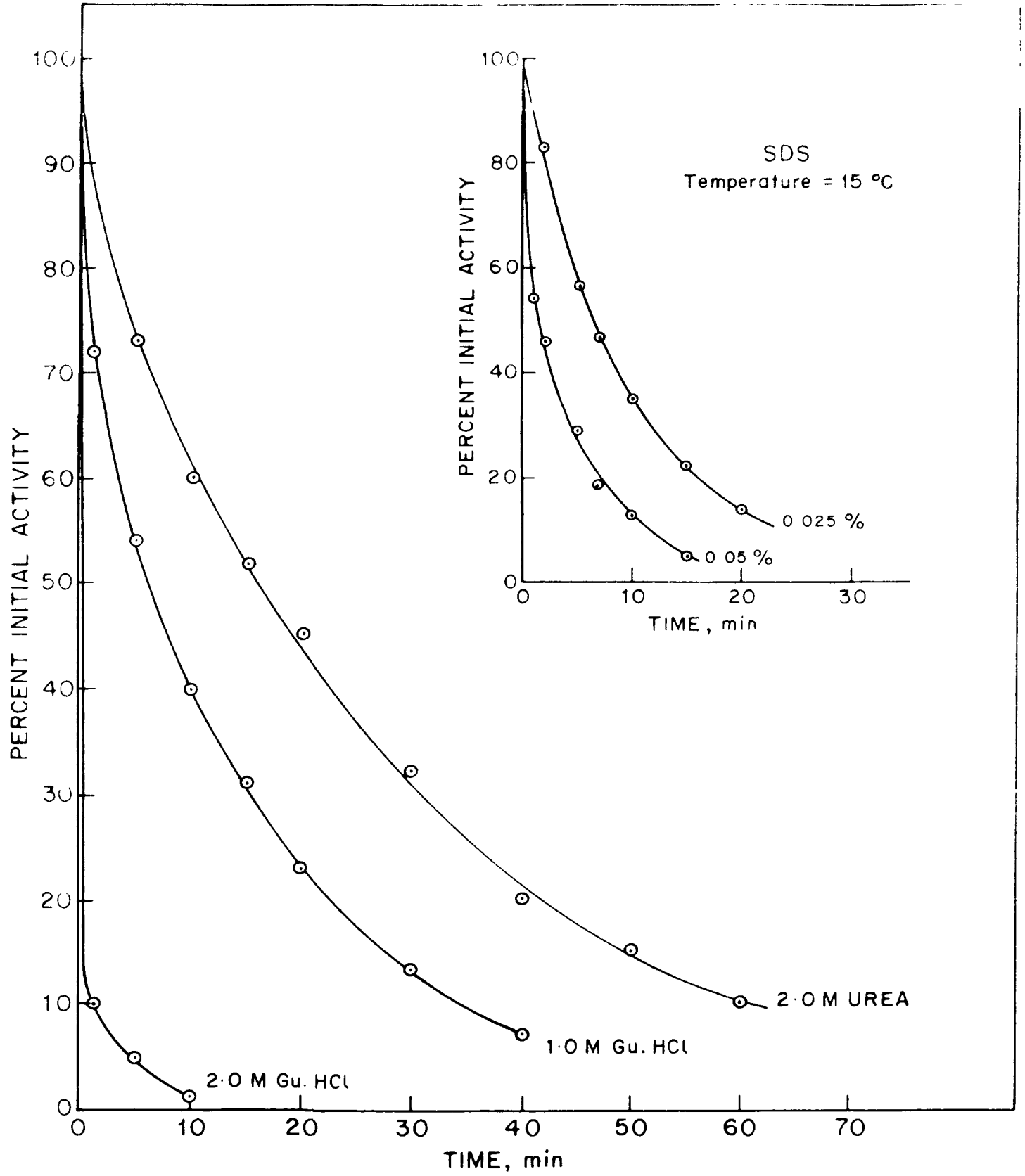


FIG. 25 TIME COURSE OF INACTIVATION OF NITRITE REDUCTASE BY UREA, Gu. HCl AND SDS.

The enzyme (4 μg) in 0·8 ml of 0·1M phosphate buffer (pH 6·8) was incubated at 5 °C with various concentrations of urea and Gu HCl. At the times indicated suitable aliquots were withdrawn and assayed for the enzyme activity. Insert. Conditions during treatment of the enzyme with SDS were same as in urea and Gu. HCl-treatment except that the incubation with SDS was carried out at 15 °C.

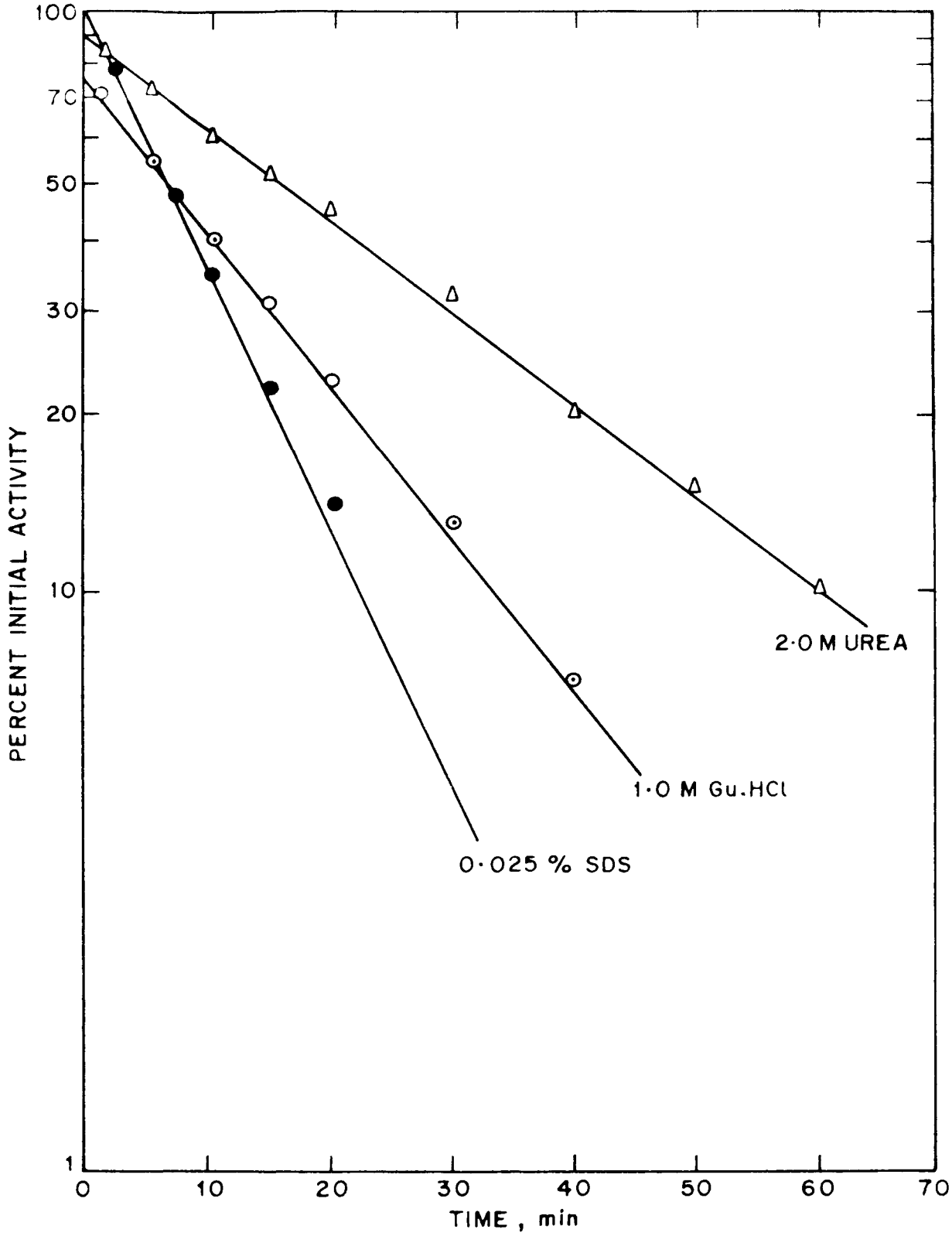


FIG 26 TIME DEPENDENCE OF UREA, Gu HCl AND SDS-INACTIVATION OF THE ENZYME

Log percent initial activities were plotted against time Data were taken from Fig. 25

Effect of temperature on enzyme activity in the presence and absence of urea

The heat-inactivation of the enzyme (in the absence of urea) at temperatures between 32 and 40°C is shown in Fig. 27. At 40°C, the enzyme loses about 90% of its activity in about 8 min. The loss in activity can be approximated by first order kinetics (Fig. 27). From the plot (Fig. 28) of the logarithm of the rate constants obtained at 32, 35, 38, and 40°C versus the reciprocal of absolute temperature, the ΔH_a for the inactivation process is calculated to be 60.1 kcal.

The heat sensitivity of the enzyme in urea was studied by incubating the enzyme in 2 M urea at various temperatures and determining the loss of activity at different intervals (Fig. 29). Ten minutes incubation (prior-incubation system) in 2 M urea at 5, 15, 20, and 25°C resulted in 40, 58, 68, and 86% loss of the enzyme activity respectively. The ΔH_a calculated for the inactivation of the enzyme in 2.0 M urea was calculated to be 9.2 kcal (Fig. 28). This value is considerably lower than that obtained for the thermal denaturation of the enzyme in the absence of urea (60.1 kcal).

Effect of protein concentration on urea- and heat-inactivation of the enzyme

The effect of enzyme concentration on urea-inactivation of the enzyme was studied. A 100-fold change of the enzyme concentration in the range of 10 μg to 1 mg/ml was found to have no significant effect. In contrast to urea-inactivation,

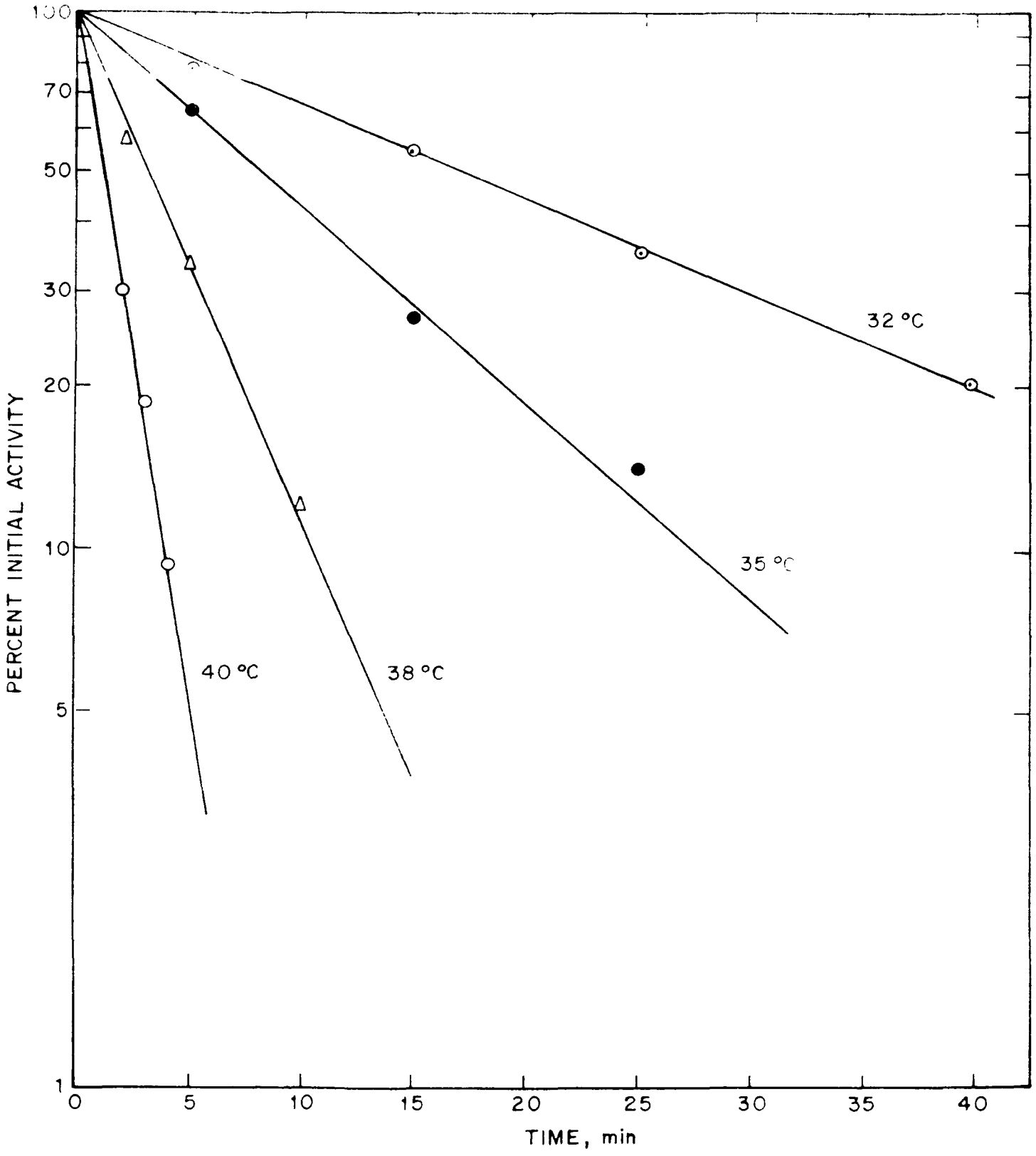


FIG. 27 TIME COURSE OF THERMAL INACTIVATION OF A FISCHERI NITRITE REDUCTASE ACTIVITY AT 32, 35, 38 AND 40 °C. Enzyme samples (4 μg) in 0 1 M PO_4 buffer (pH 6·8) were incubated in a water bath at each temperature. Suitable aliquots were withdrawn and assayed for residual activities at the intervals indicated

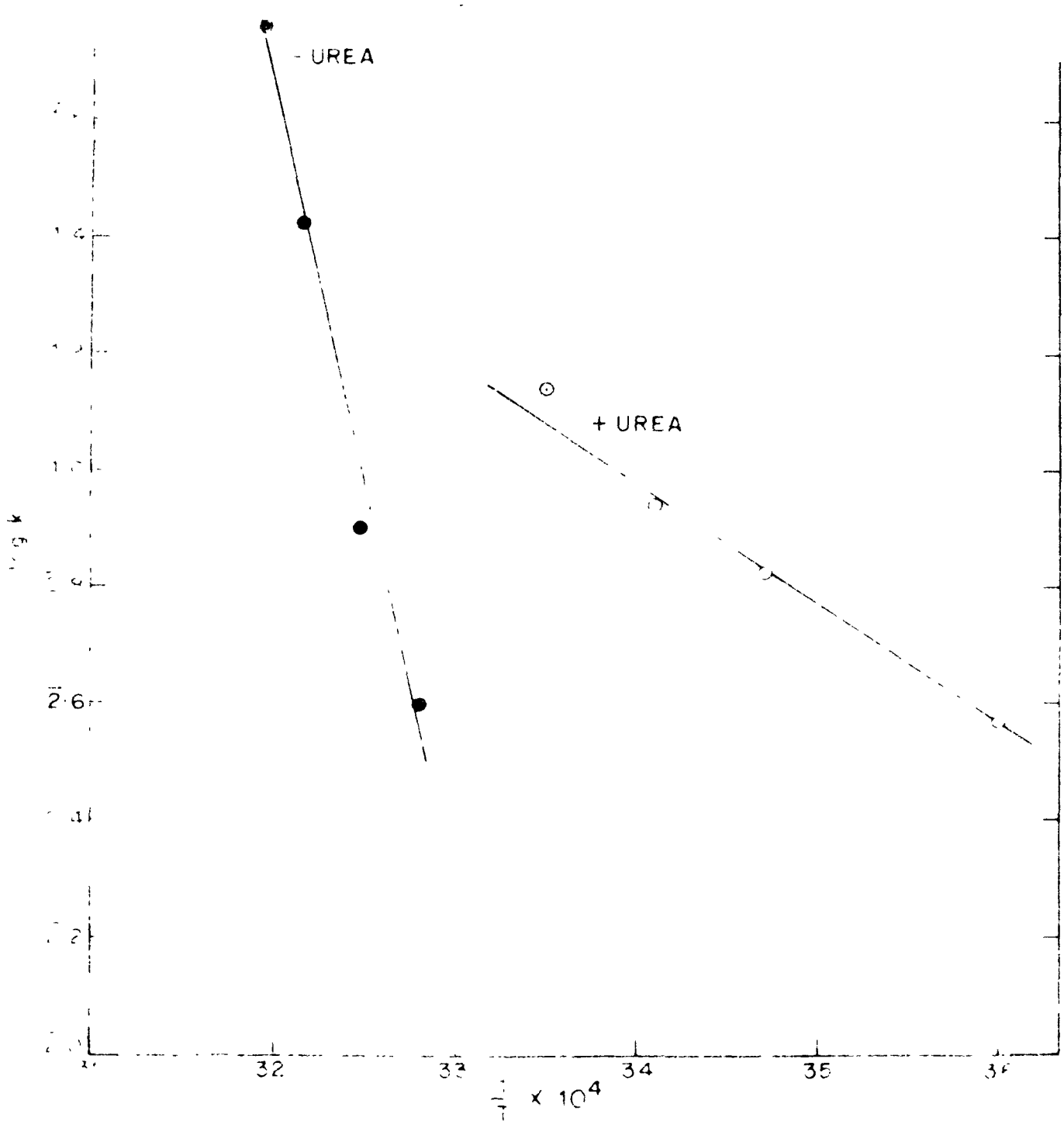


FIG 28 DETERMINATION OF ENERGIES OF ACTIVATION FOR HEAT- AND UREA (2M) - INACTIVATION OF A. FISCHERI NITRITE REDUCTASE

The data of figs 27 and 28 were used

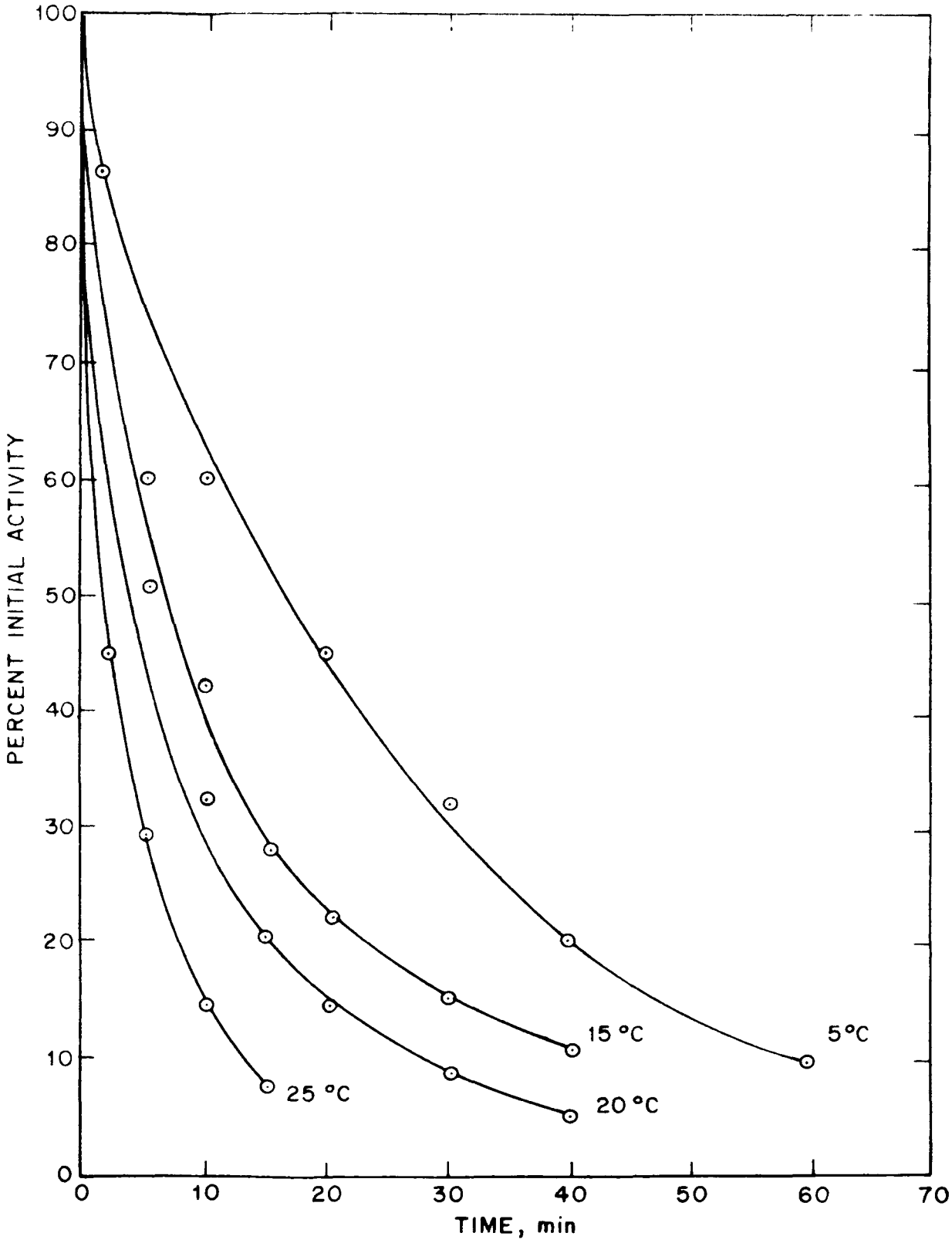


FIG. 29 EFFECT OF TEMPERATURE ON THE INACTIVATION OF NITRITE REDUCTASE WITH 2 M UREA.

Enzyme (4 μg) in 0·1 M phosphate buffer (pH 6·8) containing 2 M urea was incubated at temperatures indicated Aliquots were taken at different intervals and assayed for the residual activities.

however, heat-inactivation was found to be dependent on protein concentration. Fig. 30 shows the effect of enzyme concentration and BSA on heat-inactivation of the enzyme. The rate of inactivation at an enzyme concentration of 10 μg/ml was much higher as compared to that at 1 mg/ml. At low concentrations, the enzyme was greatly stabilized towards heat-inactivation by BSA (1 mg/ml). The mechanism by which BSA influences the enzyme stability is not understood. The stabilizing effect of BSA following heat and acid treatment is probably a reflection of its ability to prevent changes in the secondary and tertiary structures of the enzyme molecule.

Effect of pH

The ꝛ inactivation of A. fischeri nitrite reductase in 2.0 M urea was determined in the pH range from 5.5 to 8.5. In the presence of urea the enzyme shows maximum stability around pH 8.0 (Fig. 31) compared to 6.5 in the absence of urea (170).

Effect of phosphate concentration

The effect of phosphate on urea-inactivation was studied by incubating the enzyme with varying concentrations of phosphate buffer, pH 6.8, containing 2 M urea. Samples were withdrawn after 1, 5, and 10 min of incubation and assayed for the residual activity. As apparent from the results presented in Table 22, the concentration of phosphate in the incubation

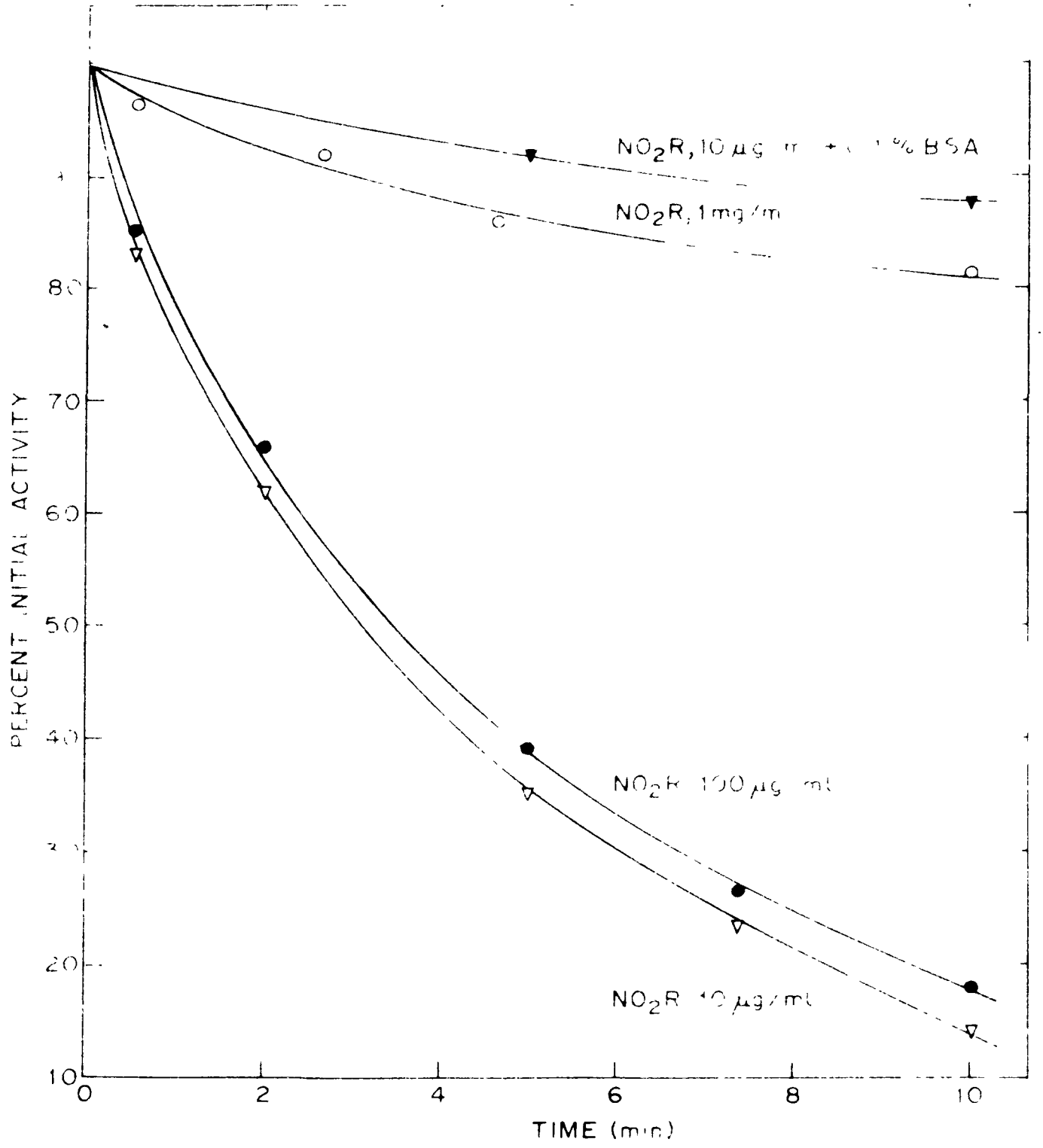


FIG 30 EFFECT OF ENZYME CONCENTRATION AND BSA ON HEAT-INACTIVATION OF THE ENZYME

The enzyme at concentrations of 10, 100 µg, and 1 mg/ml was incubated at 35 °C in 0.1 M phosphate buffer, pH 6.8. Residual activities were determined at intervals indicated. In another experiment, 10 µg of enzyme was incubated under similar conditions in the presence of 0.1% BSA

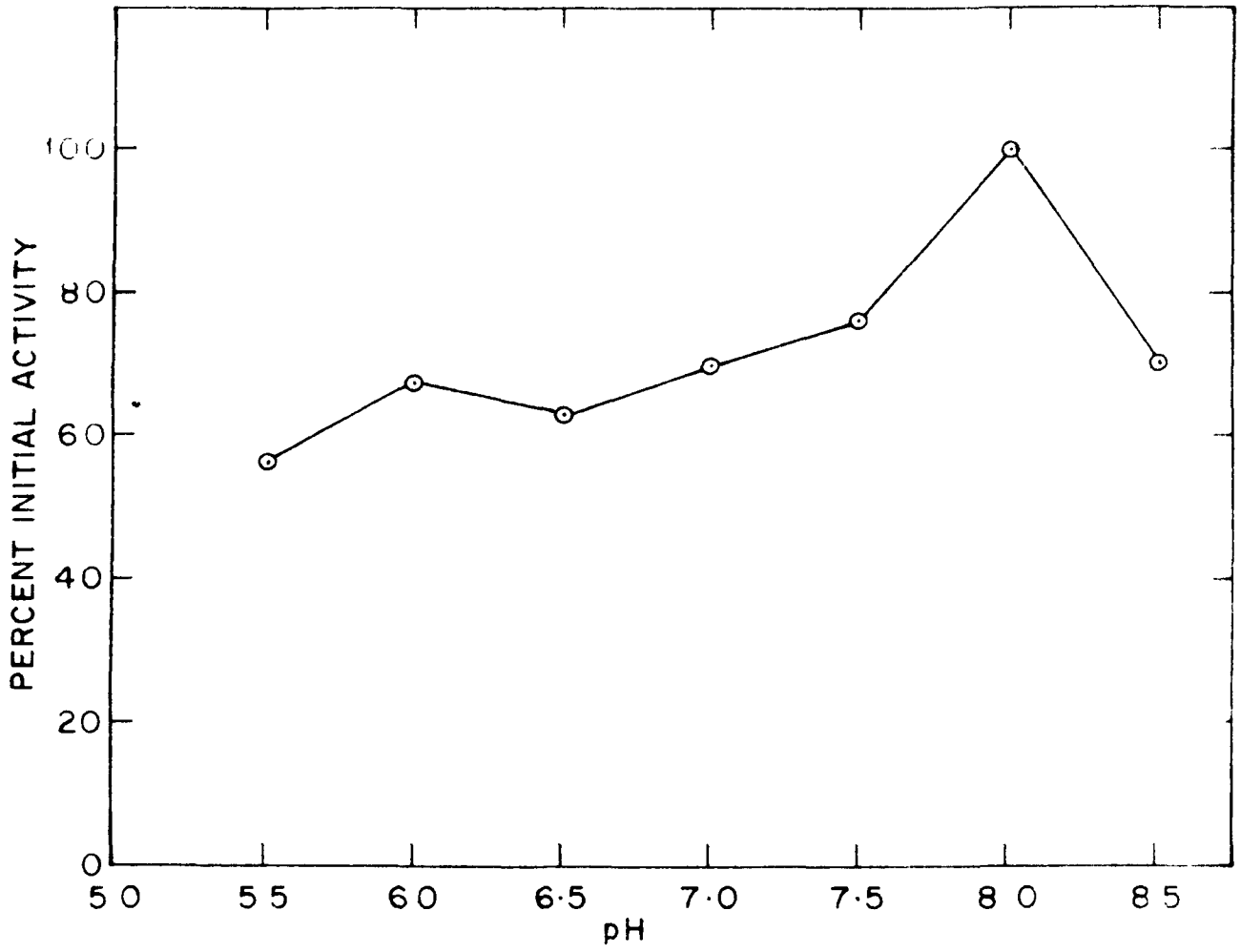


FIG 31 EFFECT OF pH ON UREA-INACTIVATION OF NITRITE REDUCTASE. The enzyme (5 μg) was incubated at 5 °C with 2 M urea in the presence of 0·1 M potassium phosphate at different pH values indicated. The residual activities were determined after 10 min of incubation

TABLE 22

Effect of phosphate concentration on the inactivation of nitrite reductase by 2M urea

4 μg of enzyme in potassium phosphate buffer of pH 6.8 at indicated concentrations was incubated with 2M urea at 5°C. Assays were carried out with suitable aliquots after 1,5, and 10 min of incubation.

Phosphate concentration (M)	Percent initial activity		
	After 1 min	After 5 min	After 10 min
0.005	20	12	5
0.050	50	35	25
0.100	70	55	40
0.200	90	80	68
0.500	100	95	90
1.000	100	100	100

medium had a profound effect on urea-inactivation of the enzyme. Thus, while 90% of the original activity was lost by 10 min incubation in 0.005 M phosphate, no loss was observed in 1.0 M phosphate. However, there was a complete loss of activity when the enzyme was treated with 4-6 M urea even in the presence of 1.0 M phosphate.

Effect of thiol-compounds

The effect of thiol-containing compounds, 2-ME, and dithiothreitol, was studied. These reagents were found to have no effect on urea-inactivation of the enzyme.

Effect of substrates

Fig. 32 illustrates that the substrates, nitrite and hydroxylamine, considerably protect the enzyme from urea-inactivation. The effect of nitrite was studied at different concentrations. The maximum protection by nitrite was obtained at a concentration of 5 mM or above. At a concentration of 0.2 mM or less, no effect of nitrite could be noticed. Hydroxylamine also protected the enzyme from urea-inactivation but it was less effective as compared to nitrite; maximum protection was obtained at a concentration of 20 mM. The order of effectiveness of nitrite and hydroxylamine against urea-inactivation does not reflect their binding affinities for the enzyme. The binding affinity for nitrite is 100 times greater than that for hydroxylamine as indicated by their Km values (170). Protective effects by substrates and coenzymes as well as

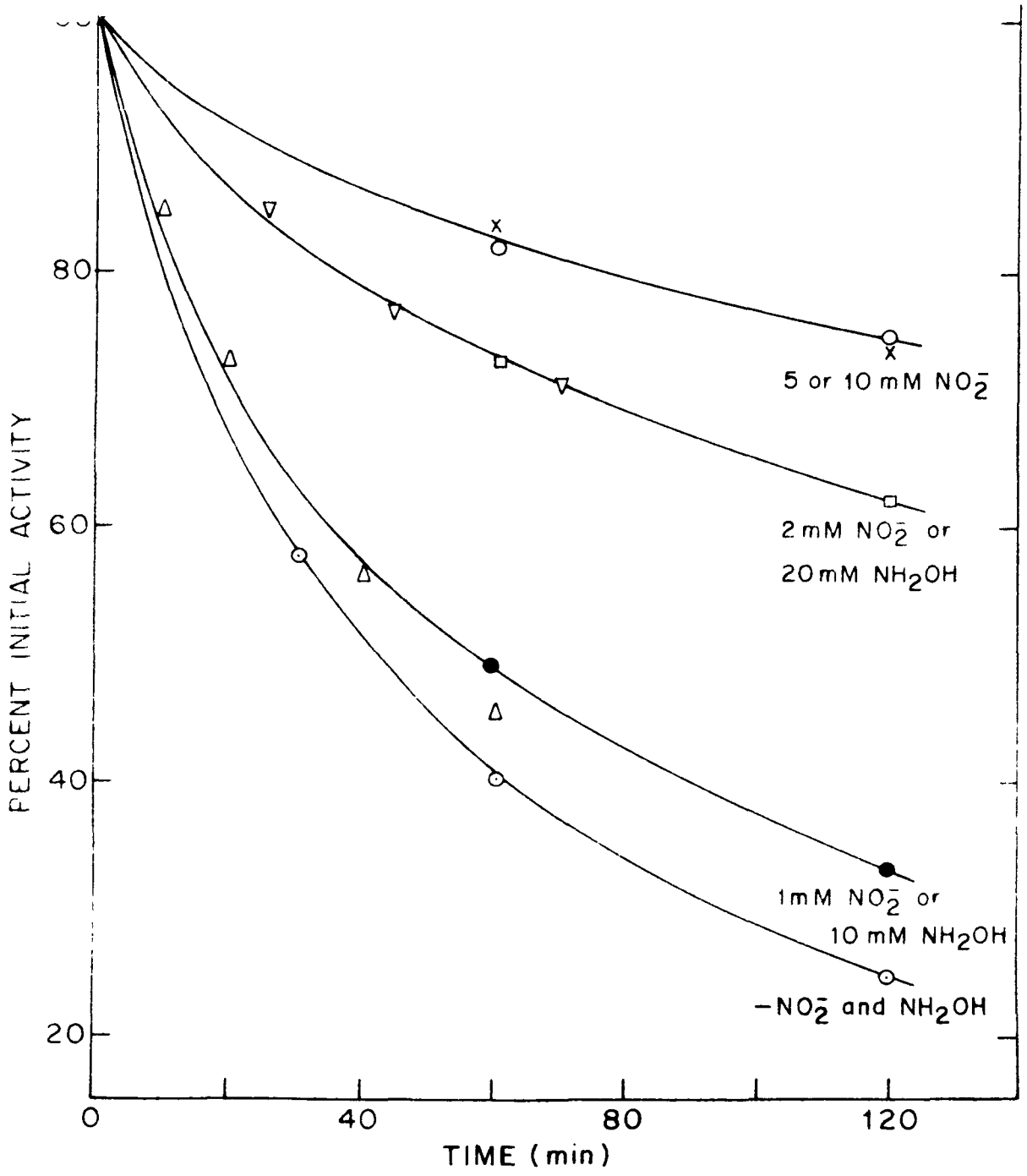


FIG 32 EFFECT OF SUBSTRATES ON THE INACTIVATION OF ENZYME BY UREA.

Enzyme samples (10 μg) in 0·1 M potassium phosphate buffer (pH 6·8) containing 2 M urea were incubated at 3 °C in the absence and presence of different concentrations of substrates. Aliquots were withdrawn at suitable intervals and assayed for the residual activities ○ = no NO_2^- and NH_2OH, ● = 1 mM NO_2^-, □ = 2 mM NO_2^-, x = 5 mM NO_2^-, o = 10 mM NO_2^-, Δ = 10 mM NH_2OH, ∇ = 20 mM NH_2OH.

inhibitors against denaturation of several enzymes have been reported (365-367).

Reversibility of the enzyme inactivation by protein denaturants

The inactivation of A. fischeri nitrite reductase with urea and Gu. HCl was found to be reversible in that the enzyme regained its activity when the denaturants were removed by dialysis against 0.05 M potassium phosphate buffer, pH 6.8. While almost complete reversal of the urea-inactivated enzyme was achieved, Gu.HCl-inactivated enzyme could be reactivated only to about 50-60%. In these reactivation experiments, the enzyme was inactivated to 95%-99%. Prolonged exposure (8 hr at 3-5°C) of the enzyme to 2-4 M urea and 1-2 M Gu.HCl, however, resulted in irreversible inactivation. The enzyme treated for a period of 5-10 min with 6-8 M urea or 4-6 M Gu.HCl also failed to regain the activity.

Reversal of urea and Gu.HCl-inactivated enzyme was also attempted by dilution. 20 to 100-fold dilution of the denatured enzyme in phosphate buffer under various conditions such as the presence of BSA, various amounts of 2-ME, changes in pH, temperature, and concentration of phosphate buffer failed to renature the enzyme.

SDS-treated enzyme failed to regain activity when the denaturant was diluted or removed by dialysis or by chilling (to precipitate SDS), suggesting that the inhibition by SDS is qualitatively different from that produced by urea and Gu.HCl. Results of reactivation experiments with urea,

Gu.HCl, and SDS are presented in Table 23.

Acid-inactivation of A. fischeri nitrite reductase and its reversal on neutralization

Nitrite reductase from A. fischeri is a relatively stable enzyme. However, incubation of the enzyme at low pH values leads to loss of the enzyme activity that can subsequently be recovered on neutralization. Fig. 33 shows the inactivation observed at pH 4.5 and 4.8 as a function of time and also the protective effect of BSA on acid inactivation. The rates of inactivation at pH above 5.0 were too slow and below pH 4.5 too fast to be measured accurately. Acid-inactivation of the enzyme, like urea-, Gu.HCl-, and SDS-inactivation, follows first order kinetics; a straight line was obtained by plotting the logarithm of the residual activity versus time (Fig. 33, insert). BSA at a concentration of 1 mg/ml protected the enzyme from acid inactivation.

The inactivation of enzyme at pH 4.8 was reversible. About 70-80% recovery was obtained by adding to inactivated enzyme equal volume of 0.2 M potassium phsophate buffer (pH 6.8) containing 0.1% BSA and incubating the mixture for about 3-5 hr at 10-15°C. With the pure enzyme, the presence of BSA is an absolute requirement for the reversal of the activity.

During reactivation experiments, it was observed that the rate and extent of reactivation depended critically upon the length of time the enzyme was exposed to acid pH. In order

TABLE 23

Reversal of urea- and guanidine hydrochloride-inactivated nitrite reductase

Inactivation with urea and Gu.HCl was carried out at 5°C in 0.005 M potassium phosphate buffer, pH 6.8. Dialysis was carried out for 18-24 hr at 2-3°C against potassium phosphate buffer, pH 6.8 with three changes of buffer. Inactivation with SDS and its removal by dialysis was carried out at 15°C as SDS tends to precipitate at lower temperatures.

Treatment	% Initial activity
a) 20 min with 2-4 M urea	0-5
b) 20 min with 2-4 M urea followed by dialysis against phosphate	90-100
c) 20 min with 1-2 M Gu.HCl	0-5
d) 20 min with 1-2 M Gu.HCl followed by dialysis against phosphate	50-60
e) 8 hr with 2-4 M urea or 1-2 M Gu.HCl followed by dialysis against phosphate	0
f) 5-10 min with 6-8 M urea or 4-6 Gu.HCl followed by dialysis against phosphate	0
g) 20 min with 0.05% SDS	0
h) 20 min with 0.05% SDS followed by dialysis against phosphate	0

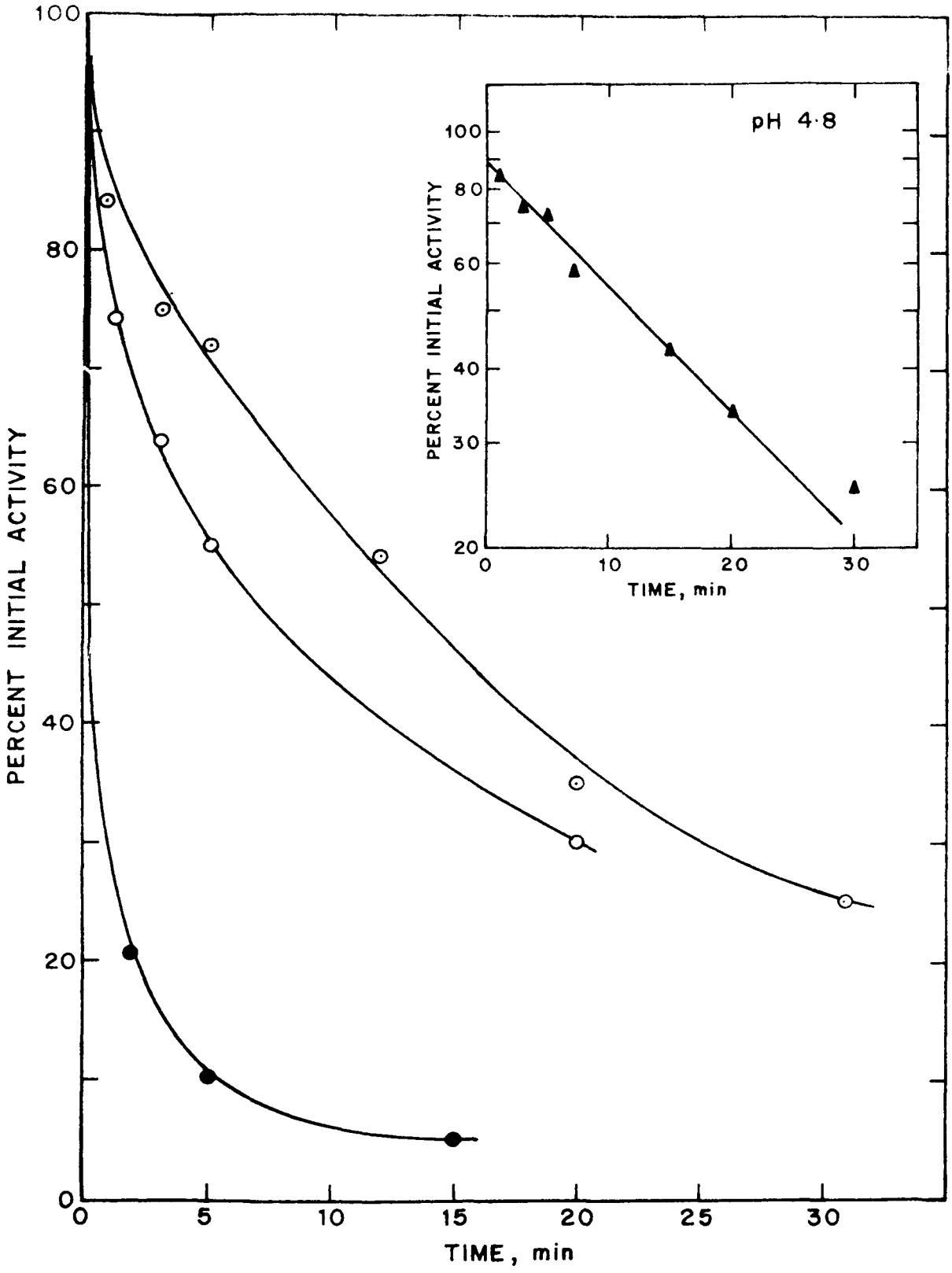


FIG. 33 INACTIVATION OF A. FISCHERI NITRITE REDUCTASE AT pH 4·5 & 4·8.

Enzyme (5μg/ml) was incubated at 3–5 °C in acetate adjusted to pH 4·5 ~~and~~ or 4·8. At the times indicated aliquots were withdrawn and assayed for the residual activity: ● = pH 4·5, no BSA; ○ = pH 4·5 + BSA (2 mg/ml); ⊙ = pH 4·8, no BSA

Insert: SEMILOGARITHMIC PLOT OF PERCENT RESIDUAL ACTIVITY VERSUS TIME AT pH 4·8.

to assess the effect of time of exposure of the enzyme at pH 4.8, the following experiment was performed. The samples of nitrite reductase (5 μg protein) were rapidly adjusted to pH 4.8 with predetermined volumes of 0.1 M acetate buffer (pH 4.7). At suitable intervals, the samples were diluted with equal volumes of 0.2 M potassium phosphate buffer (pH 6.8). The final pH of the reactivation mixture was 6.5. The return of enzymic activity at 15°C was monitored by withdrawing samples and assaying the activity. The results are shown in Fig. 34. It is apparent that both the rate and extent of reactivation were considerably influenced by the length of time the enzyme had remained at pH 4.8.

Characteristics of the renatured enzyme

It was of interest to determine if the renatured enzyme was similar to the native enzyme. The Km values of the renatured nitrite reductase (urea, and acid-treated) were similar to that of the native enzyme (7-9 x 10^{-5}M at pH 7.5).

A. fischeri nitrite reductase also catalyzes the reduction of hydroxylamine (170). The renatured enzyme also shows hydroxylamine reductase activity. The ratio of nitrite to hydroxylamine reductase activities for the renatured enzyme was also the same as that of the native enzyme (9.0 to 9.5). These results suggest that the structural changes caused by acid, and urea-treatments are reversible.

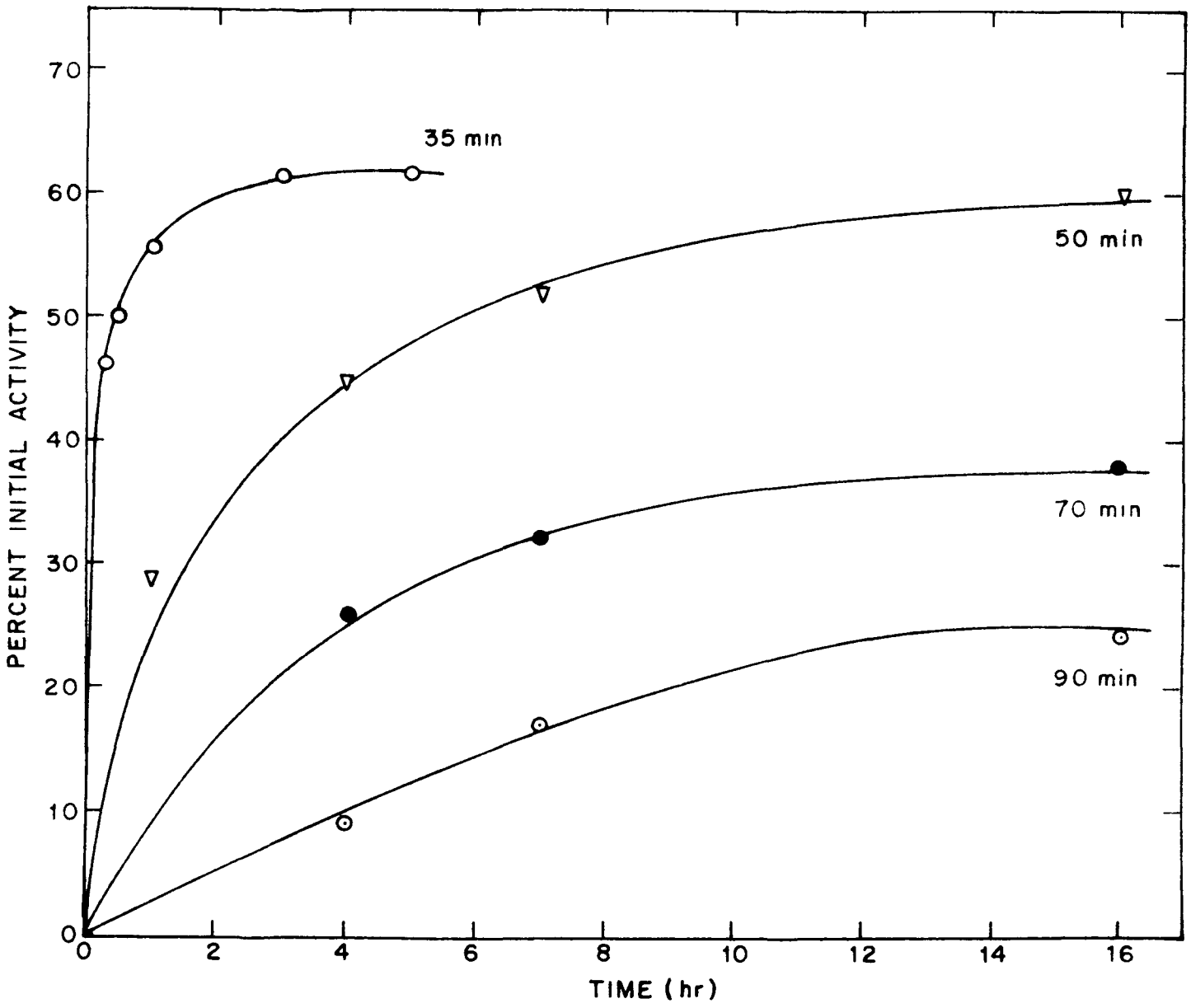


FIG. 34 RATE AND EXTENT OF RECOVERY OF ENZYMIC ACTIVITY FOLLOWING INACTIVATION AT pH 4·8.

Enzyme samples (10 μg/ml) were inactivated at pH 4·8 (0·1 acetate) and 5 °C for different time intervals (35 to 90 min). Reactivation was initiated by addition, to the acidified enzyme samples, of an equal volume of 0·2 M potassium phosphate buffer (pH 6·8) containing 0·1 % BSA at 15 °C.

DISCUSSION

The present investigation demonstrates that A. fischeri nitrite reductase is markedly inactivated by urea, Gu.HCl, and SDS at relatively low concentrations as well as on incubation at acid pH.

Phosphate at a concentration of 0.5-1.0 M greatly stabilizes the enzyme against urea inactivation. Multivalent anions, sulfate and phosphate, are reported to protect the native conformation of macromolecules as diverse as collagen, ribonuclease, myosin, and DNA (368).

The substrates, nitrite and hydroxylamine, considerably protect the enzyme from inactivation by 2 M urea; nitrite is more effective than hydroxylamine. BSA also protected the enzyme against inactivation. Substrates and cofactors are known to function as stabilizers (365-367) or destabilizers (369,370) for their respective enzymes. The stabilizing effect of phosphate and the other compounds is probably a reflection of their abilities to protect changes in the secondary and tertiary structure of proteins.

The type of Ackermann and Potter plot for the inhibition of the enzyme by urea would indicate that the inhibition is of pseudo-irreversible type. Analysis of the inactivation by the method of Johnson, Eyring, and Williams (363) indicated that 2 molecules of urea are involved in the formation of enzyme-urea complex which leads to the observed inhibition. This suggested

a specific effect of urea within a small region of the enzyme molecule. However, inhibition by urea with respect to nitrite is of noncompetitive type. The noncompetitive kinetics of the inhibition by urea suggest that the site of attachment of urea is other than the active site. The fact that protection from urea-inactivation required concentrations of nitrite of about 70-80 x Km together with the noncompetitive mode of inhibition suggests that urea induces structural changes.

The results presented in this chapter and those of chapter IV rule out the possibility that inactivation by urea, Gu.HCl, and acid involves dissociation of the enzyme into its subunits.

The inactivation could largely be reversed on removal of urea and Gu.HCl by dialysis or on neutralization of acid. Inactivation by SDS, however, results in irreversible loss of enzyme activity indicating that the action of SDS is qualitatively different from that of urea and Gu.HCl. Similar observations have been made by Chilson *et al.* (356).

For reactivation to occur on removal of urea and Gu.HCl by dialysis, it was necessary that the period required to produce complete inactivation be restricted to the minimum. Exposure of the enzyme to these denaturants for longer periods results in irreversible loss of the activity. All attempts to renature the urea-, and Gu.HCl-inactivated enzyme by dilution under different environmental conditions were unsuccessful. The failure to obtain reversal of enzyme activity on dilution

could mean that the dissociation constant of urea-enzyme complex is very small. The inactivation of the enzyme at pH 4.8 is also reversible. About 70-80% of the original activity is recovered on neutralization. The presence of BSA in the reactivation mixture is an absolute requirement for renaturation. An absolute requirement of glycerol, another stabilizing agent, for the reactivation of spinach leaf glyoxalic acid reductase has been reported by Kohn (371). He also showed that the presence of BSA in the reactivation mixture greatly increased the yield of the enzyme activity.

The observation that the rate and extent of reactivation is dependent upon the length of time the enzyme was exposed to acid pH is similar to that reported by Anderson and Weber (37[illegible] for lactate dehydrogenase and by Mann and Vestling (373) for malate dehydrogenase. Chilson *et al.* (350) have, however, reported that neither the rate nor the degree of reactivation of pig heart malate dehydrogenase was influenced by the period the enzyme was exposed to acid pH.

By the criteria of Km and the ratio of nitrite to hydroxylamine reductase activities, the acid-, and urea-inactivated-renatured enzymes were indistinguishable from the native enzyme.

Chapter 7

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The *Achromobacter fischeri* nitrite reductase which catalyzes the reduction of nitrite to ammonia, a six-electron reduction, has been obtained for the first time in a homogeneous form as judged by ultracentrifugation and disc gel electrophoresis. The overall recovery of the enzyme is 31%. The purified enzyme has a specific activity of 150-155 μmoles NO_2^- reduced per min per mg protein with reduced benzyl viologen as an electron donor. This is the highest specific activity reported hitherto for any nitrite reductase. Reduced methyl viologen also serves as an electron donor and is twice as effective as benzyl viologen.

The enzyme has an average molecular weight of 80,000 daltons as determined by the Archibald approach-to-equilibrium method, disc gel electrophoresis, and from a combination of the hydrodynamic properties. A significantly low value of 66,000-67,000 daltons is determined from gel chromatography on Sephadex G-200 and Bio-gel P-150 according to the procedure of Andrews. The Stokes' radius of the enzyme determined from the gel filtration data is 3.49 nm. Combination of Stokes' radius and $s^\circ_{20,w}$ yields a molecular weight of 78,000. The agreement between this value and that obtained by other methods lends support to Siegel and Monty's suggestion (274) that it is the molecular radius and not the molecular weight which determines the behaviour of macromolecules upon gel filtration.

The other physico-chemical properties of the enzyme determined from sedimentation analysis and gel filtration experiments are as follows: the $s^{\circ}_{20,w}$ is 5.25 S; the diffusion constant, 6.05 F; frictional ratio, 1.25, and the axial ratio, 3.0. The sedimentation coefficient exhibits a slight dependence on protein concentration.

The enzyme does not dissociate in the presence of 6 M guanidine hydrochloride (Gu.HCl) or 6 M urea. The enzyme, however, splits into subunits upon treatment with 6 M Gu.HCl or 1% sodium dodecyl sulfate (SDS) in combination with 1% 2-mercaptoethanol (2-ME). The sedimentation coefficient of the enzyme subunits determined in 6 M Gu.HCl-0.1 M 2-ME system at a protein concentration of 7 mg/ml is 1.4 S. The presence of a single symmetrical peak in the ultracentrifuge suggests that the subunits are apparently of identical size. The molecular weight of the subunits is 38,000 to 39,000 daltons as determined by the Archibald approach-to-equilibrium method in 6 M Gu.HCl-0.1 M 2-ME system and SDS-gel electrophoresis.

The amino acid analyses indicate an amino acid composition for a total of 693 amino acid residues of: Trp_8, $1/2\text{-}Cys_{10}$, Met_{17}, Arg_{18}, His_{22}, Pro_{24}, Tyr_{26}, Phe_{34}, Val_{41}, Gly_{42}, Ser_{42}, Ile_{44}, Thr_{44}, Leu_{48}, Lys_{50}, Ala_{52}, Asp_{85}, Glu_{86}, and amide $ammonia_{62}$. The analysis shows a predominance of lysine, alanine, aspartic acid and glutamic acid residues whereas those of tryptophan, 1/2-cystine, methionine, and arginine are low in number.

The results of tyrosine and tryptophan determinations by spectrophotometric and colorimetric methods are in good agreement with each other and with the tyrosine content determined in the Amino acid analyzer.

Titration of the enzyme with p-HMB or DTNB shows that the enzyme contains 4 free sulfhydryl groups. The finding of the same value in the presence or absence of denaturing agents indicates that all the free -SH groups are accessible for reaction with DTNB or p-HMB. The value of 6 -SH groups per mole of enzyme obtained after $NaBH_4$ reduction followed by DTNB titration indicates the presence of one -S-S- linkage. The value of 10 moles of cysteic acid per mole of enzyme obtained on performic acid-oxidized protein also indicates the presence of one -S-S- linkage assuming that 4 -SH groups are involved in thioether linkages for binding the two heme moieties present in the enzyme molecule, each of the heme being bound by two thioether linkages.

Titration of all the four free -SH groups detected in the nitrite reductase molecule has no effect on the enzyme activity suggesting that the -SH groups are not involved in enzyme action. The inhibition of the enzyme activity at relatively high concentration is not related to mercaptide formation but involves other non-specific interactions. The reversal of p-HMB or p-CMB inhibition by -SH-containing compounds suggests that no permanent derangement in the enzyme structure is induced by the sulfhydryl reagents.

Dansylation studies indicate methionine as the only N-terminal residue. The failure to find any N-terminal amino acid

other than methionine, and the identical molecular weight of the two subunits suggest that the two subunits are similar though not necessarily identical.

The data obtained indicate that A. fischeri nitrite reductase is composed of two subunits of equivalent size which are covalently bonded by a disulfide bridge.

The partial specific volume and isoionic point of the enzyme calculated from the amino acid composition are 0.73 ml/g and 5.1 respectively. The degree of hydrophobicities of the A. fischeri enzyme in terms of average hydrophobicity (HO_{av}), polarity index (p), and the frequency of non-polar side chains (NPS) are 1075 cal/residue, 1.00 and 0.32 respectively. These are in good agreement with the values calculated for the nitrite reductases from P. aeruginosa and C. fusca.

A. fischeri nitrite reductase is markedly inactivated at acid pH and on treatment with urea, Gu.HCl, and SDS at relatively low concentrations. Gu.HCl is more effective on a molar basis than urea. Phosphate, nitrite, and hydroxylamine markedly protect the enzyme from inactivation by 2 M urea. The optimal concentrations of phosphate and the substrates for the protection of enzyme against urea-inactivation are 0.5 M, 5 mM, and 20 mM respectively.

The kinetic analyses show that the inactivation involves 2 moles of urea per mole of enzyme and is of non-competitive type with a Ki of 1.45-1.6 M. The ΔH_a for the inactivation of the enzyme in 2 M urea is 9.2 kcal compared to

60 kcal obtained in the absence of urea.

The inactivation of the enzyme by 2-4 M urea and 1-2 M Gu.HCl is reversible. As much as 90 to 95% of the initial activity is recovered when urea is removed by dialysis. Only 50 to 60% reversal is achieved with Gu.HCl-treated enzyme. Inactivation of the enzyme with SDS is, however, irreversible suggesting that the action of SDS is qualitatively different from that of urea and Gu.HCl.

The inactivation of the enzyme at acid pH is also reversed on neutralization. About 70 to 75% reversal is achieved under optimal conditions. The presence of bovine serum albumin at a concentration of 1.0 mg/ml in the reactivation mixture is an absolute requirement for the reversal. The rate and extent of the reactivation depend upon the length of time the enzyme is exposed to acid, pH.

The K_m, and the ratio of nitrite reductase to hydroxylamine reductase activities of the renatured enzyme are same as that of the native enzyme.

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